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(54) Title: COMPOSITIONS AND METHODS FOR ANTIGEN-SPECIFIC VACCINATION

(57) Abstract

The present invention provides methods and compositions for inducing a prophylactic immune response to a self-antigen in a subject.

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## COMPOSITIONS AND METHODS FOR ANTIGEN-SPECIFIC VACCINATION

### CROSS-REFERENCE TO RELATED APPLICATIONS

5 This application claims priority under 35 U.S.C. § 119(e) of U.S. Provisional Application No. 60/078,890, filed March 20, 1998, the contents of which are hereby incorporated by reference into the present disclosure.

### TECHNICAL FIELD

10 This invention is in the field of molecular immunology and medicine. In particular, the present invention provides compositions and methods for inducing an immune response to a native self-antigen in a subject.

### BACKGROUND

15 The goal of vaccination is to generate a protective immune response and an expanded population of memory cells ready to encounter the infectious agent, which will then elicit a potent secondary immune response. T and B cells are highly antigen specific and can develop into memory cells, and therefore are the target for a successful vaccine.

20 Tumor specific T cells, derived from cancer patients, will bind and lyse tumor cells. This specificity is based on their ability to recognize short amino acid sequences (epitopes) presented on the surface of the tumor cells by MHC class I and class II molecules. These epitopes are derived from the proteolytic degradation of intracellular proteins called tumor antigens encoded by genes that are either uniquely or aberrantly expressed in tumor or cancer cells.

25 The availability of specific anti-tumor T cells has enabled the identification of tumor antigens and subsequently the generation of cancer vaccines designed to provoke an anti-tumor immune response. A critical target of

vaccines is the specialized antigen-presenting cell ("APC"), the most immunologically powerful of which is the bone marrow-derived dendritic cell ("DC").

5           Cancer vaccines have met with limited success. Since many naturally occurring neoplasms express only non-mutated self antigens, it is hypothesized that these antigens cannot serve as inducers and/or targets for a tumor destructive immune response, although immunological reactions mediated by either lymphocytes or antibodies to cultivated human tumors have been reported.

10           Hellstrom and Hellstrom (1969) *Adv. Cancer Res.* **12**:167-223. Mechanisms of systemic immune tolerance to self have begun to emerge, particularly from studies in transgenic mouse systems. Hanahan (1990) *Ann. Rev. Cell Biol.* **6**:493-537. Mechanisms of systemic immune tolerance include deletion of potentially autoreactive B or T cells, induction of anergy in B and T cells, and the poorly defined phenomenon of suppression of immune response by suppressor cells.

15           Houghton and Lewis, pages 37-54 in Forni et al. eds. (1994) *CYTOKINE-INDUCED TUMOR IMMUNOGENICITY*, Academic Press, New York. Thus, a need exists to overcome immune tolerance to self-antigens and to provide an effective cancer vaccine. This invention satisfies these needs and provides related advantages as well.

20           DISCLOSURE OF THE INVENTION  
25           This invention relates to the use of antigens for prophylactic vaccination against disease for which an immune response to the encoded antigen would be therapeutically effective in preventing or delaying the onset of the disease or to prolong a disease-free period in the subject.

In one aspect, this invention provides a substantially pure population of educated, antigen-specific immune effector cells produced by stimulating naïve immune effector cells with antigen presenting cells (APCs) that present a self-antigen or an altered self-antigen.

In another aspect, this invention provides a method of inducing an immune response to a self-antigen in a subject, comprising administering to the subject an effective amount of APCs presenting the self-antigen or an altered self-antigen. In one embodiment, the cells have been genetically modified to express and present the antigen.

5 In a further aspect, this invention provides a method of inducing an immune response to a self-antigen in a subject, comprising administering to the subject an effective amount of the self-antigen or an altered self-antigen to the subject. In one embodiment, a polynucleotide encoding the antigen or altered antigen is administered to the subject.

#### BRIEF DESCRIPTION OF THE FIGURES

10 Figure 1 (SEQ ID NO:1 through SEQ ID NO:5) depicts the murine and human amino acid sequences for the melanoma antigen gp100. Minimal essential epitopes are identified in bold lettering. The coding sequences can be deduced from these amino acid sequences.

15 Figure 2 (SEQ ID NO:6 through SEQ ID NO:13) depicts the coding sequence for human and murine melanoma antigen MART1.

20 Figure 3 (SEQ ID NO:14 through SEQ ID NO:15) depicts the coding sequence for the human antigen MART1 and its deduced amino acid sequence.

Figure 4 (SEQ ID NO:16 through SEQ ID NO:17) is the coding sequence for the human tyrosinase-related-protein-2 cDNA. CTL epitopes are underlined.

25 Figure 5 shows the results of an assay of CTLs generated from normal donor PBLs with adenoviral gp100 infected dendritic cells.

#### MODES FOR CARRYING OUT THE INVENTION

Throughout this disclosure, various publications, patents and published patent specifications are referenced by an identifying citation. The disclosures of these publications, patents and published patent specifications are hereby

incorporated by reference into the present disclosure to more fully describe the state of the art to which this invention pertains.

*Definitions*

5            The practice of the present invention will employ, unless otherwise indicated, conventional techniques of immunology, molecular biology, microbiology, cell biology and recombinant DNA, which are within the skill of the art. See, e.g., Sambrook, Fritsch and Maniatis, MOLECULAR CLONING: A LABORATORY MANUAL, 2<sup>nd</sup> edition (1989); CURRENT PROTOCOLS IN  
10          MOLECULAR BIOLOGY (F. M. Ausubel et al. eds., (1987)); the series METHODS IN ENZYMOLOGY (Academic Press, Inc.); PCR 2: A PRACTICAL APPROACH (M.J. MacPherson, B.D. Hames and G.R. Taylor eds. (1995)), Harlow and Lane eds. (1989) ANTIBODIES, A LABORATORY MANUAL, and ANIMAL CELL CULTURE (R.I. Freshney ed. (1987)).

15          As used herein, certain terms may have the following defined meanings.

As used in the specification and claims, the singular form "a", "an" and "the" include plural references unless the context clearly dictates otherwise. For example, the term "a cell" includes a plurality of cells, including mixtures thereof.

20          The term "genetically modified" means containing and/or expressing a foreign gene or nucleic acid sequence which in turn, modifies the genotype or phenotype of the cell or its progeny. In other words, it refers to any addition, deletion or disruption to a cell's endogenous nucleotides. Methods and compositions useful in genetic modification are described in detail below.

25          The terms "antigen-presenting cells" or "APCs" includes both intact, whole cells as well as other molecules which are capable of inducing the presentation of one or more antigens, preferably in association with class I MHC molecules. Examples of suitable APCs are discussed in detail below and include, but are not limited to, whole cells such as macrophages, dendritic cells, B cells, purified MHC class I molecules complexed to 92-microglobulin, foster antigen presenting cells, hybrid APCs and pulsed APCs.

Dendritic cells (DCs) are potent antigen-presenting cells (APCs). It has been shown that DCs provide all the signals required for T cell activation and proliferation. These signals can be categorized into two types. The first type, which gives specificity to the immune response, is mediated through interaction between the T-cell receptor/CD3 ("TCR/CD3") complex and an antigenic peptide presented by a major histocompatibility complex ("MHC") class I or II protein on the surface of APCs. This interaction is necessary, but not sufficient, for T cell activation to occur. In fact, without the second type of signals, the first type of signals can result in T cell anergy. The second type of signals, called co-stimulatory signals, is neither antigen-specific nor MHC-restricted, and can lead to a full proliferation response of T cells and induction of T cell effector functions in the presence of the first type of signals.

As used herein, "dendritic cell" is to include, but not be limited to a pulsed dendritic cell, a foster cell or a dendritic cell hybrid. It also is intended to encompass dendritic cells from any source, *e.g.*, human, murine or simian.

The term "antigen" is well understood in the art and includes substances which are immunogenic, *i.e.*, immunogens, as well as substances which induce immunological unresponsiveness, or anergy, *i.e.*, anergens. As used herein, the term "antigen" is intended to mean full length proteins as well as peptide fragments thereof containing or comprising epitope.

A "native", "natural" or "wild-type" antigen is a polypeptide, protein or a fragment which contains an epitope, which has been isolated from a natural biological source, and which can specifically bind to an antigen receptor, in particular a T cell antigen receptor (TCR), in a subject.

An altered antigen is one having a primary sequence that is different from that of the corresponding wild-type antigen. Altered antigens can be made by synthetic or recombinant methods and include, but are not limited to antigenic peptides that are differentially modified during or after translation, *e.g.*, by phosphorylation, glycosylation, crosslinking, acylation, proteolytic cleavage, linkage to an antibody molecule, membrane molecule or other ligand. (Ferguson

et al. (1988) Ann. Rev. Biochem. 57:285-320). A synthetic or altered antigen of the invention is intended to bind to the same TCR as the natural epitope.

5 A “self-antigen” also referred to herein as a native or wild-type antigen is an antigenic peptide that induces little or no immune response in the subject due to self-tolerance to the antigen. An example of a self-antigen is the human melanoma antigen gp100.

10 The term “immune effector cells” refers to cells capable of binding an antigen and which mediate an immune response. These cells include, but are not limited to, T cells, B cells, monocytes, macrophages, NK cells and cytotoxic T lymphocytes (CTLs), for example CTL lines, CTL clones, and CTLs from tumor, inflammatory, or other infiltrates. Certain diseased tissue expresses specific antigens and CTLs specific for these antigens have been identified. For example, approximately 80% of melanomas express the antigen known as GP-100.

15 The term “immune effector molecule” as used herein, refers to molecules capable of antigen-specific binding, and includes antibodies, T cell antigen receptors, and MHC Class I and Class II molecules.

A “naïve” cell, *e.g.*, a naïve immune effector cell, is a cell that has never been exposed to an antigen.

20 As used herein, the term “educated, antigen-specific immune effector cell” is an immune effector cell as defined above, which has encountered antigen and which is specific for that antigen. An educated, antigen-specific immune effector cell may be activated upon binding antigen. “Activated” implies that the cell is no longer in  $G_0$  phase, and begins to produce cytokines characteristic of the cell type. For example, activated  $CD4^+$  T cells secrete IL-2 and have a higher number of 25 high affinity IL-2 receptors on their cell surfaces relative to resting  $CD4^+$  T cells.

30 A peptide or polypeptide of the invention may be preferentially recognized by antigen-specific immune effector cells, such as B cells and T cells. In the context of T cells, the term “recognized” intends that a peptide or polypeptide of the invention, comprising one or more antigenic epitopes, is recognized, *i.e.*, is presented on the surface of an APC together with (*i.e.*, bound to) an MHC

5

molecule in such a way that a T cell antigen receptor (TCR) on the surface of an antigen-specific T cell binds to the epitope wherein such binding results in activation of the T cell. The term "preferentially recognized" intends that a polypeptide of the invention is substantially not recognized, as defined above, by a T cell specific for an unrelated antigen. Assays for determining whether an epitope is recognized by an antigen-specific T cell are known in the art and are described herein.

10

The term "autogeneic" or "autologous" as used herein, indicates the origin of a cell. Thus, a cell being administered to an individual (the "recipient") is autogeneic if the cell was derived from that individual (the "donor") or a genetically identical individual. An autogeneic cell can also be a progeny of an autogeneic cell. The term also indicates that cells of different cell types are derived from the same donor or genetically identical donors.

15

The term "culturing" refers to the *in vitro* propagation of cells or organisms on or in media of various kinds. It is understood that the descendants of a cell grown in culture may not be completely identical (either morphologically, genetically, or phenotypically) to the parent cell. By "expanded" is meant any proliferation or division of cells.

20

A "subject" is a vertebrate, preferably a mammal, more preferably a human. Mammals include, but are not limited to, murines, simians, humans, farm animals, sport animals, and pets.

25

As used herein "expression" refers to the process by which polynucleotides are transcribed into mRNA and translated into peptides, polypeptides, or proteins. If the polynucleotide is derived from genomic DNA, expression may include splicing of the mRNA, if an appropriate eukaryotic host is selected. Regulatory elements required for expression include promoter sequences to bind RNA polymerase and transcription initiation sequences for ribosome binding. For example, a bacterial expression vector includes a promoter such as the *lac* promoter and for transcription initiation the Shine-Dalgarno sequence and the start codon AUG (Sambrook et al. (1989) *supra*). Similarly, an

30

5 eukaryotic expression vector includes a heterologous or homologous promoter for RNA polymerase II, a downstream polyadenylation signal, the start codon AUG, and a termination codon for detachment of the ribosome. Such vectors can be obtained commercially or assembled by the sequences described in methods well known in the art, for example, the methods described below for constructing vectors in general.

10 The terms "major histocompatibility complex" or "MHC" refers to a complex of genes encoding cell-surface molecules that are required for antigen presentation to T cells and for rapid graft rejection. In humans, the MHC complex is also known as the HLA complex. The proteins encoded by the MHC complex are known as "MHC molecules" and are classified into class I and class II MHC molecules. Class I MHC molecules include membrane heterodimeric proteins made up of an  $\alpha$  chain encoded in the MHC associated noncovalently with  $\beta 2$ -microglobulin. Class I MHC molecules are expressed by nearly all nucleated 15 cells and have been shown to function in antigen presentation to  $CD8^+$  T cells. Class I molecules include HLA-A, -B, and -C in humans. Class II MHC molecules also include membrane heterodimeric proteins consisting of noncovalently associated  $\alpha$  and  $\beta$  chains. Class II MHC are known to participate 20 in antigen presentation to  $CD4^+$  T cells and, in humans, include HLA-DP, -DQ, and DR. The term "MHC restriction" refers to a characteristic of T cells that permits them to recognize antigen only after it is processed and the resulting antigenic peptides are displayed in association with either a self class I or class II MHC molecule. Methods of identifying and comparing MHC are well known in the art and are described in Allen et al. (1994) Human Immunol. **40**:25-32; 25 Santamaria et al. (1993) Human Immunol. **37**:39-50; and Hurley et al. (1997) Tissue Antigens **50**:401-415.

30 An "isolated" or "purified" population of cells is substantially free of cells and materials with which it is associated in nature. By substantially free or substantially purified is meant at least 50% of the population are immune effector cells, preferably at least 70%, more preferably at least 80%, and even more

preferably at least 90% free of non-immune effector cells with which they are associated in nature.

“Operably linked” refers to a juxtaposition wherein the elements are in an arrangement allowing them to function.

5 A “gene delivery vehicle” is defined as any molecule that can carry inserted polynucleotides into a host cell. Examples of gene delivery vehicles are liposomes, biocompatible polymers, including natural polymers and synthetic polymers; lipoproteins; polypeptides; polysaccharides; lipopolysaccharides; artificial viral envelopes; metal particles; and bacteria, viruses, such as 10 baculovirus, adenovirus and retrovirus, bacteriophage, cosmid, plasmid, fungal vectors and other recombination vehicles typically used in the art which have been described for expression in a variety of eukaryotic and prokaryotic hosts, and may be used for gene therapy as well as for simple protein expression.

15 A “viral vector” is defined as a recombinantly produced virus or viral particle that comprises a polynucleotide to be delivered into a host cell, either *in vivo*, *ex vivo* or *in vitro*. Examples of viral vectors include retroviral vectors, adenovirus vectors, adeno-associated virus vectors and the like. In aspects where gene transfer is mediated by a retroviral vector, a vector construct refers to the 20 polynucleotide comprising the retroviral genome or part thereof, and a therapeutic gene. As used herein, “retroviral mediated gene transfer” or “retroviral transduction” carries the same meaning and refers to the process by which a gene or nucleic acid sequences are stably transferred into the host cell by virtue of the virus entering the cell and integrating its genome into the host cell genome. The virus can enter the host cell via its normal mechanism of infection or be modified 25 such that it binds to a different host cell surface receptor or ligand to enter the cell. As used herein, retroviral vector refers to a viral particle capable of introducing exogenous nucleic acid into a cell through a viral or viral-like entry mechanism.

Retroviruses carry their genetic information in the form of RNA; however, once the virus infects a cell, the RNA is reverse-transcribed into the DNA form

which integrates into the genomic DNA of the infected cell. The integrated DNA form is called a provirus.

In aspects where gene transfer is mediated by a DNA viral vector, such as an adenovirus (Ad) or adeno-associated virus (AAV), a vector construct refers to the polynucleotide comprising the viral genome or part thereof, and a transgene. Adenoviruses (Ads) are a relatively well characterized, homogenous group of viruses, including over 50 serotypes. (see, e.g., WO 95/27071). Ads are easy to grow and do not require integration into the host cell genome. Recombinant Ad-derived vectors, particularly those that reduce the potential for recombination and generation of wild-type virus, have also been constructed. (see, WO 95/00655; WO 95/11984). Wild-type AAV has high infectivity and specificity integrating into the host cell's genome. Hermonat and Muzyczka (1984) PNAS USA **81**:6466-6470 and Lebkowski et al. (1988) Mol. Cell. Biol. **8**:3988-3996.

Vectors that contain both a promoter and a cloning site into which a polynucleotide can be operatively linked are well known in the art. Such vectors are capable of transcribing RNA *in vitro* or *in vivo*, and are commercially available from sources such as Stratagene (La Jolla, CA) and Promega Biotech (Madison, WI). In order to optimize expression and/or *in vitro* transcription, it may be necessary to remove, add or alter 5' and/or 3' untranslated portions of the clones to eliminate extra, potential inappropriate alternative translation initiation codons or other sequences that may interfere with or reduce expression, either at the level of transcription or translation. Alternatively, consensus ribosome binding sites can be inserted immediately 5' of the start codon to enhance expression.

Gene delivery vehicles also include several non-viral vectors, including DNA/liposome complexes, and targeted viral protein-DNA complexes. Liposomes that also comprise a targeting antibody or fragment thereof can be used in the methods of this invention. To enhance delivery to a cell, the nucleic

acid or proteins of this invention can be conjugated to antibodies or binding fragments thereof which bind cell surface antigens, e.g., TCR, CD3 or CD4.

“Hybridization” refers to a reaction in which one or more polynucleotides react to form a complex that is stabilized via hydrogen bonding between the bases of the nucleotide residues. The hydrogen bonding may occur by Watson-Crick base pairing, Hoogstein binding, or in any other sequence-specific manner. The complex may comprise two strands forming a duplex structure, three or more strands forming a multi-stranded complex, a single self-hybridizing strand, or any combination of these. A hybridization reaction may constitute a step in a more extensive process, such as the initiation of a PCR reaction, or the enzymatic cleavage of a polynucleotide by a ribozyme.

Examples of stringent hybridization conditions include: incubation temperatures of about 25°C to about 37°C; hybridization buffer concentrations of about 6 X SSC to about 10 X SSC; formamide concentrations of about 0% to about 25%; and wash solutions of about 6 X SSC. Examples of moderate hybridization conditions include: incubation temperatures of about 40°C to about 50°C; buffer concentrations of about 9 X SSC to about 2 X SSC; formamide concentrations of about 30% to about 50%; and wash solutions of about 5 X SSC to about 2 X SSC. Examples of high stringency conditions include: incubation temperatures of about 55°C to about 68°C; buffer concentrations of about 1 X SSC to about 0.1 X SSC; formamide concentrations of about 55% to about 75%; and wash solutions of about 1 X SSC, 0.1 X SSC, or deionized water. In general, hybridization incubation times are from 5 minutes to 24 hours, with 1, 2, or more washing steps, and wash incubation times are about 1, 2, or 15 minutes. SSC is 0.15 M NaCl and 15 mM citrate buffer. It is understood that equivalents of SSC using other buffer systems can be employed.

A polynucleotide or polynucleotide region (or a polypeptide or polypeptide region) has a certain percentage (for example, 80%, 85%, 90%, or 95%) of “sequence identity” to another sequence means that, when aligned, that percentage of bases (or amino acids) are the same in comparing the two

sequences. This alignment and the percent homology or sequence identity can be determined using software programs known in the art, for example those described in CURRENT PROTOCOLS IN MOLECULAR BIOLOGY (F.M. Ausubel et al., eds., 1987) Supplement 30, section 7.7.18, Table 7.7.1. Preferably, default parameters are used for alignment. A preferred alignment program is BLAST, using default parameters. In particular, preferred programs are BLASTN and BLASTP, using the following default parameters: Genetic code = standard; filter = none; strand = both; cutoff = 60; expect = 10; Matrix = BLOSUM62; Descriptions = 50 sequences; sort by = HIGH SCORE; Databases = non-redundant, GenBank + EMBL + DDBJ + PDB + GenBank CDS translations + SwissProtein + SPupdate + PIR. Details of these programs can be found at the following Internet address: <http://www.ncbi.nlm.nih.gov/cgi-bin/BLAST>.

“Co-stimulatory molecules” are involved in the interaction between receptor-ligand pairs expressed on the surface of antigen presenting cells and T cells. Research accumulated over the past several years has demonstrated convincingly that resting T cells require at least two signals for induction of cytokine gene expression and proliferation (Schwartz, R.H. (1990) *Science* **248**:1349-1356 and Jenkins, M.K. (1992) *Immunol. Today* **13**:69-73). One signal, the one that confers specificity, can be produced by interaction of the TCR/CD3 complex with an appropriate MHC/peptide complex. The second signal is not antigen specific and is termed the "co-stimulatory" signal. This signal was originally defined as an activity provided by bone-marrow-derived accessory cells such as macrophages and dendritic cells, the so called "professional" APCs. Several molecules have been shown to enhance co-stimulatory activity. These are heat stable antigen (HSA) (Liu Y. et al. (1992) *J. Exp. Med.* **175**:437-445); chondroitin sulfate-modified MHC invariant chain (Ii-CS) (Naujokas M.F. et al. (1993) *Cell* **74**:257-268); intracellular adhesion molecule 1 (ICAM-1) (Van Seventer G.A. (1990) *J. Immunol.* **144**:4579-4586); and B7-1 and B7-2/B70 (Schwartz R.H. (1992) *Cell* **71**:1065-1068). Co-stimulatory molecules are commercially available from a variety of sources, including, for example,

Beckman Coulter. It is intended, although not always explicitly stated, that molecules having similar biological activity as wild-type or purified co-stimulatory molecules (e.g., recombinantly produced or muteins thereof) are intended to be used within the spirit and scope of the invention.

5 As used herein, the term "inducing an immune response in a subject" is a term well understood in the art and intends that an increase of at least about 2-fold, more preferably at least about 5-fold, more preferably at least about 10-fold, more preferably at least about 100-fold, even more preferably at least about 500-fold, even more preferably at least about 1000-fold or more in an immune  
10 response to an antigen (or epitope) can be detected (measured), after introducing the antigen (or epitope) into the subject, relative to the immune response (if any) before introduction of the antigen (or epitope) into the subject. An immune response to an antigen (or epitope), includes, but is not limited to, production of an antigen-specific (or epitope-specific) antibody, and production of an immune  
15 cell expressing on its surface a molecule which specifically binds to an antigen (or epitope). Methods of determining whether an immune response to a given antigen (or epitope) has been induced are well known in the art. For example, antigen-specific antibody can be detected using any of a variety of immunoassays known in the art, including, but not limited to, ELISA, wherein, for example, binding of  
20 an antibody in a sample to an immobilized antigen (or epitope) is detected with a detectably-labeled second antibody (e.g., enzyme-labeled mouse anti-human Ig antibody).

25 The term "immune effector molecule", as used herein, refers to molecules capable of antigen-specific binding, and includes antibodies, T cell antigen receptors, and MHC Class I and Class II molecules.

30 As used herein, the terms "neoplastic cells", "neoplasia", "tumor", "tumor cells", "cancer" and "cancer cells", (used interchangeably) refer to cells which exhibit relatively autonomous growth, so that they exhibit an aberrant growth phenotype characterized by a significant loss of control of cell proliferation (i.e., de-regulated cell division). Neoplastic cells can be malignant or benign.

“Suppressing” tumor growth indicates a growth state that is curtailed when compared to growth without contact with educated, antigen-specific immune effector cells described herein. Tumor cell growth can be assessed by any means known in the art, including, but not limited to, measuring tumor size, determining whether tumor cells are proliferating using a  $^3\text{H}$ -thymidine incorporation assay, or counting tumor cells. “Suppressing” tumor cell growth means any or all of the following states: slowing, delaying, and stopping tumor growth, as well as tumor shrinkage.

The term “culturing” refers to the *in vitro* propagation of cells or organisms on or in media of various kinds. It is understood that the descendants of a cell grown in culture may not be completely identical (morphologically, genetically, or phenotypically) to the parent cell. By “expanded” is meant any proliferation or division of cells.

As used herein, the term “cytokine” refers to any one of the numerous factors that exert a variety of effects on cells, for example, inducing growth or proliferation. Non-limiting examples of cytokines which may be used alone or in combination in the practice of the present invention include, interleukin-2 (IL-2), stem cell factor (SCF), interleukin 3 (IL-3), interleukin 6 (IL-6), interleukin 12 (IL-12), G-CSF, granulocyte macrophage-colony stimulating factor (GM-CSF), interleukin-1 alpha (IL-1 $\alpha$ ), interleukin-11 (IL-11), MIP-1 $\alpha$ , leukemia inhibitory factor (LIF), c-kit ligand, thrombopoietin (TPO) and flt3 ligand. The present invention also includes culture conditions in which one or more cytokine is specifically excluded from the medium. Cytokines are commercially available from several vendors such as, for example, Genzyme (Framingham, MA), Genentech (South San Francisco, CA), Amgen (Thousand Oaks, CA), R&D Systems and Immunex (Seattle, WA).

As used herein, an “immunostimulatory agent” is any agent that enhances or supplements the immune response, *e.g.*, a cytokine or co-stimulatory molecule.

A “composition” is intended to mean a combination of active agent and another compound or composition, inert (for example, a detectable agent or label) or active, such as an adjuvant.

5 A “pharmaceutical composition” is intended to include the combination of an active agent with a carrier, inert or active, making the composition suitable for diagnostic or therapeutic use *in vitro*, *in vivo* or *ex vivo*.

10 As used herein, the term “pharmaceutically acceptable carrier” encompasses any of the standard pharmaceutical carriers, such as a phosphate buffered saline solution, water, and emulsions, such as an oil/water or water/oil emulsion, and various types of wetting agents. The compositions also can include stabilizers and preservatives. For examples of carriers, stabilizers and adjuvants, see Martin REMINGTON'S PHARM. SCI., 15th Ed. (Mack Publ. Co., Easton (1975)).

15 An “effective amount” is an amount sufficient to effect beneficial or desired results. An effective amount can be administered in one or more administrations, applications or dosages. In the context of a disease state, an effective amount of an immunomodulatory agent of the invention, including a peptide of the invention, a polynucleotide of the invention, an educated, antigen-specific immune effector cell and/or an APC of the invention, is an amount that is sufficient to palliate, ameliorate, stabilize, reverse, slow or delay the progression 20 of the disease state.

25 As used herein, the term “comprising” is intended to mean that the compositions and methods include the recited elements, but not excluding others. “Consisting essentially of” when used to define compositions and methods, shall mean excluding other elements of any essential significance to the combination. Thus, a composition consisting essentially of the elements as defined herein would not exclude trace contaminants from the isolation and purification method and pharmaceutically acceptable carriers, such as phosphate buffered saline, preservatives, and the like. “Consisting of” shall mean excluding more than trace elements of other ingredients and substantial method steps for administering the

compositions of this invention. Embodiments defined by each of these transition terms are within the scope of this invention.

5 This invention provides cancer vaccines and methods of using the vaccines to induce a prophylactic immune response to a native self-antigen in a subject.

For purposes of immunization, the self-antigen (usually a tumor-associated antigen or TAA) can be delivered to antigen-presenting cells as protein/peptide or, in the form of cDNA encoding the protein/peptide. As used herein, the self-antigens include altered forms of the antigen (defined above) that bind to the same epitope as the native or wild-type self-antigen. The antigens may be homologous 10 or heterologous to the cell on which it is presented.

APCs may be genetically modified *ex vivo* or targeted *in vivo* using the vectors described herein. Antigen-presenting cells (APCs) can consist of dendritic cells (DCs), monocytes/macrophages, B lymphocytes or other cell 15 type(s) expressing the necessary MHC/co-stimulatory molecules. The APCs may be derived from any species, *e.g.*, murine, human or simian, and may be autologous or heterologous to a subject.

The methods described below focus primarily on DCs which are the most potent, preferred APCs. The term "antigen" is used in its broadest sense and 20 includes minimal epitopes and chimeric molecules in addition to isolated full length proteins. As noted above, the antigen of this vaccine is homologous, heterologous (*e.g.*, a murine antigen administered to a human patient) or an altered antigen as compared to the corresponding native self-antigen. Antigens can be antigens of any type, *e.g.*, tumor associated antigens.

25 For the purpose of illustration only, the polynucleotides encoding TAAs of this invention can be, in one embodiment, previously characterized tumor-associated antigens such as gp100 (Kawakami et al. (1997) *Intern. Rev. Immunol.* **14**:173-192); MUC-1 (Henderson et al. (1996) *Cancer Res.* **56**:3763-3770); MART-1 (Kawakami et al. (1994) *Proc. Natl. Acad. Sci.* **91**:3515-3519; 30 Kawakami et al. (1997) *Intern. Rev. Immunol.* **14**:173-192; Ribas et al. (1997)

Cancer Res. **57**:2865-2869); HER-2/neu (U.S. Patent No. 5,550,214); MAGE (PCT/US92/04354); HPV16, 18E6 and E7 (Ressing et al. (1996) Cancer Res. **56**(1):582-588; Restifo (1996) Current Opinion in Immunol. **8**:658-663; Stern (1996) Adv. Cancer Res. **69**:175-211; Tindle et al. (1995) Clin. Exp. Immunol. **101**:265-271; and van Driel et al. (1996) Annals of Medicine **28**:471-477); CEA (U.S. Patent No. 5,274,087); PSA (Lundwall A. (1989) Biochem. Biophys. Research Communications **161**:1151-59); prostate membrane specific antigen (PSMA) (Israeli et al. (1993) Cancer Research **53**:227-30); tyrosinase (U.S. Patent Nos. 5,530,096 and 4,898,814; Brichard et al. (1993) J. Exp. Med. **178**:489-49); tyrosinase related proteins 1 or 2 (TRP-1 and TRP-2); NY-ESO-1 (Chen et al. (1997) Proc. Natl. Acad. Sci. U.S.A. **94**:1914-18); or the GA733 antigen (U.S. Patent No. 5,185,254). Sequences for exemplary antigens are provided in Figures 1 through 4. The human and murine MUC1 coding sequences are provided under Genbank Accession No. M35093 and M64928.

It is known within the state of the art that minor modification to a nucleotide sequence will not affect the function of the molecules encoded thereby. Thus, biologically equivalent polynucleotides of published sequences are also useful in the methods described herein, as well as the polypeptides encoded thereby. Indeed, the altered polypeptides are encompassed within the term “altered antigen” as used herein. These polynucleotides can be identified by hybridization under stringent conditions (defined above) to the sequences disclosed in the published references or known in the art. Alternatively, the polynucleotides and polypeptides can be identified as being at least 80%, or more preferably, at least 90% or most preferably, at least 95%, identical (defined above) to the disclosed sequences using sequence alignment programs and default parameters. Polypeptides and polynucleotides having the above noted sequence identity to native antigen are intended to be encompassed within the term “altered antigen.”

Also within the scope of this invention is an epitope or wild-type antigenic peptide corresponding to a yet unidentified protein. A common strategy in the

search for tumor antigens is to isolate tumor-specific T-cells and attempt to identify the antigens recognized by these cells. In patients with cancer, specific CTLs have been derived from lymphocytic infiltrates present at the tumor site. Weidmann et al., *supra*. These TILs are unique cell population that can be traced back to sites of disease when they are labeled with indium and adoptively transferred. Alternatively, large libraries of putative antigens can be produced and tested. Using the "phage method" (Scott and Smith (1990) *Science* **249**:386-390; Cwirla et al. (1990) *Proc. Natl. Acad. Sci.* **87**:6387-6382; and Devlin et al. (1990) *Science* **249**:404-406), very large libraries can be constructed. Another approach uses primarily chemical methods, of which the Geysen method (Geysen et al. (1986) *Mol. Immunol.* **23**:709-715 and Geysen et al. (1987) *J. Immunol. Method* **102**:259-274) and the method of Fodor et al. (1991) *Science* **251**:767-773, are examples. Furka et al. (1988) 14th Inter. Cong. Bio. Vol. **5**, Abst. FR:013; Furka (1991) *Inter. J. Peptide Protein Res.* **37**:487-493); Houghton (U.S. Patent No. 4,683,211, issued December 1986); and Rutter, et al. (U.S. Patent No. 5,010,175, issued April 23, 1991) describe methods to produce a mixture of peptides that can be tested as agonists or antagonists.

In a further aspect of this invention, Solid-PHase Epitope REcovery ("SPHERE", described in PCT WO 97/35035) can be used to identify tumor antigens and produce altered antigens or epitopes that can induce a prophylactic response against a self-antigen. SPHERE is described below.

Furthermore, the invention provides a method for cloning the cDNA and genomic DNA encoding such a protein by generating degenerate oligonucleotide probes or primers based on the sequence of the epitope. Compositions comprising the nucleic acid and a carrier, such as a pharmaceutically acceptable carrier, a solid support or a detectable label, are further provided by this method as well as methods for detecting the sequences in a sample using methods such as Northern analysis, Southern analysis and PCR.

Further provided by this invention are therapeutic and diagnostic oligopeptide sequences determined according to the foregoing methods.

Compositions comprising the oligopeptide sequence and a carrier, such as a pharmaceutically acceptable carrier, a solid support or a detectable label, are further provided by this method as well as methods for detecting the oligopeptide sequence in a sample using methods such as Western analysis and ELISA.

5 Harlow and Lane (1989), *supra*.

More specifically, this invention provides a means to elicit CD4<sup>+</sup> and CD8<sup>+</sup> T cells responses in a subject who is not exhibiting disease symptoms associated with the antigen. In other words, the methods involve eliciting a protective immune response in normal individuals that may be environmentally at 10 risk or genetically predisposed to disease. Since many known tumor antigens are non-mutated self-antigens, immunization against these antigens in normal subjects involves breaking tolerance to these self antigens. Assays to determine T cell response are well known in the art and any method that will compare T cell number prior to and subsequent to therapy can be utilized. In addition, the 15 induction of co-stimulatory cytokines by the heterologous antigen could also stimulate pre-existing anergic or low affinity self-reactive CTL clones.

When the method is practiced *ex vivo*, the APCs are manipulated to express the antigen or interest. Methods of inducing APCs to express antigens include peptide-pulsing, or, preferably, genetic modification that results in 20 expression of polynucleotides encoding the antigen of interest. When the methods are practiced *in vivo*, an effective amount of a gene delivery vehicle which expresses the antigen of interest is administered to the subject. Preferably, the 25 gene delivery vehicle is a recombinant adenoviral vector that preferentially infects APCs such as dendritic cells. Compositions comprising the modified APCs with a carrier are also provided. The methods of this invention can be further modified by co-administering an effective amount of a cytokine or co-stimulatory molecule to the subject.

In another embodiment, the APC expressing an antigen can be used to expand and isolate a population of immune effectors which, in turn, are useful for 30 adoptive immunotherapy alone or as an adjuvant to the methods described above.

As above, cytokines and/or co-stimulatory molecules or nucleic acids encoding them, can be co-administered with the immune effector cells. An increase in the T cell count as compared to prior to therapeutic administration is a positive indication that the immune effector cell is a therapeutic for use in the method.

5 Prior to administration *in vivo*, the immune effector cells are screened *in vitro* for their ability to lyse tumor cells.

Identification of antigens recognized and lysed by tumor-specific CTLs is required to identify novel antigens. The antigens of this invention can be an isolated counterpart or fragment of the counterpart to the human or animal tumor-  
10 associated antigen.

Also within the scope of this invention is an epitope or antigenic peptide corresponding to a yet unidentified protein. Thus, this invention also provides a screen to identify novel heterologous or altered antigens that induces an immune response in the subject by assaying for the ability of the putative heterologous or  
15 altered antigen for the ability to cross-react with native antigen and induce an immune response in a subject. The murine melanoma antigen gp100 and its biological activity *in vitro* and *in vivo* is a positive control in this assay. Using the methods described herein, the biological activity of the putative heterologous or altered antigen can be compared to its biological activity.

20

The following examples are provided to illustrate, but not limit the invention.

#### Materials and Methods

25

##### **Isolation, Culturing and Expansion of APCs, Including Dendritic Cells**

Dendritic cells are specialized antigen presenting cells (APCs) that are critical for eliciting T cell mediated immune responses. At least two methods have been used for the generation of human dendritic cells from hematopoietic  
30 precursor cells in peripheral blood. One approach utilizes the rare CD34+

precursor cells and stimulate them with GM-CSF plus TNF- $\alpha$ . The other method makes use of the more abundant CD34- precursor population and stimulate them with GM-CSF plus IL-4.

In one aspect of the invention, the methods described in Romani et al (1996), *infra* and Bender et al (1996) *J. Immunol. Methods* **196**:121-135, are used to generate both immature and mature dendritic cells from the peripheral blood mononuclear cells (PBMC) of a mammal, such as a murine, simian or human. Briefly, isolated PBMC are pre-treated to deplete T- and B-cells by means of an immunomagnetic technique. Lymphocyte-depleted PBMC are then cultured for 7 days in RPMI medium, supplemented with 1% autologous human plasma and GM-CSF/IL-4, to generate dendritic cells. Dendritic cells are nonadherent when compared to their monocyte progenitors. Thus, on day 7, non-adherent cells are harvested for further processing.

The dendritic cells derived from PBMC in the presence of GM-CSF and IL-4 are immature, in that they can lose the nonadherence property and revert back to macrophage cell fate if the cytokine stimuli are removed from the culture. The dendritic cells in an immature state are very effective in processing native protein antigens for the MHC class II restricted pathway (Romani et al. (1989) *J. Exp. Med.* **169**:1169.

Further maturation of cultured dendritic cells is accomplished by culturing for 3 days in a macrophage-conditioned medium (CM), which contains the necessary maturation factors. Mature dendritic cells are less able to capture new proteins for presentation but are much better at stimulating resting T cells (both CD4 $^{+}$  and CD8 $^{+}$ ) to grow and differentiate.

Older previously utilized methods involve (1) isolating bone marrow precursor cells (CD34 $^{+}$ ) from blood and stimulating them to differentiate into APC; or (2) collecting the precommitted APCs from peripheral blood. In the first approach, the patient must be treated with cytokines such as GM-CSF to boost the number of circulating CD34 $^{+}$  stem cells in the peripheral blood.

The second approach for isolating APCs is to collect the relatively large numbers of precommitted APCs already circulating in the blood. Previous techniques for isolating committed APCs from human peripheral blood have involved combinations of physical procedures such as metrizamide gradients and adherence/nonadherence steps (Freudenthal et al. (1990) PNAS **87**:7698-7702); Percoll gradient separations (Mehta-Damani et al. (1994) J. Immunol. **153**:996-1003); and fluorescence activated cell sorting techniques (Thomas et al. (1993) J. Immunol. **151**:6840-52).

One technique for separating large numbers of cells from one another is known as countercurrent centrifugal elutriation (CCE). In this technique, cells are subject to simultaneous centrifugation and a washout stream of buffer which is constantly increasing in flow rate. The constantly increasing countercurrent flow of buffer leads to fractional cell separations that are largely based on cell size.

In one aspect of the invention, the APC are precommitted or mature dendritic cells which can be isolated from the white blood cell fraction of a mammal, such as a murine, simian or a human (See, e.g., WO 96/23060). The white blood cell fraction can be from the peripheral blood of the mammal. This method includes the following steps: (a) providing a white blood cell fraction obtained from a mammalian source by methods known in the art such as leukophoresis; (b) separating the white blood cell fraction of step (a) into four or more subfractions by countercurrent centrifugal elutriation, (c) stimulating conversion of monocytes in one or more fractions from step (b) to dendritic cells by contacting the cells with calcium ionophore or GT-CSF and IL-4, (d) identifying the dendritic cell-enriched fraction from step (c), and (e) collecting the enriched fraction of step (d), preferably at about 4°C. One way to identify the dendritic cell-enriched fraction is by fluorescence-activated cell sorting. The white blood cell fraction can be treated with calcium ionophore in the presence of other cytokines, such as rhIL-12, rhGM-CSF, or rhIL-4. The cells of the white blood cell fraction can be washed in buffer and suspended in  $\text{Ca}^{++}/\text{Mg}^{++}$  free media prior to the separating step. The white blood cell fraction can be obtained

5 by leukapheresis. The dendritic cells can be identified by the presence of at least one of the following markers: HLA-DR, HLA-DQ, or B7.2, and the simultaneous absence of the following markers: CD3, CD14, CD16, 56, 57, and CD 19, 20. Monoclonal antibodies specific to these cell surface markers are commercially available.

10 More specifically, the method requires collecting an enriched collection of white cells and platelets from leukapheresis that is then further fractionated by countercurrent centrifugal elutriation (CCE) (Abrahamsen et al. (1991) *J. Clin. Apheresis* **6**:48-53). Cell samples are placed in a special elutriation rotor. The rotor is then spun at a constant speed of, for example, 3000 rpm. Once the rotor has reached the desired speed, pressurized air is used to control the flow rate of cells. Cells in the elutriator are subjected to simultaneous centrifugation and a washout stream of buffer which is constantly increasing in flow rate. This results in fractional cell separations based largely but not exclusively on differences in 15 cell size.

20 Quality control of APC and more specifically DC collection and confirmation of their successful activation in culture is dependent upon a simultaneous multi-color FACS analysis technique which monitors both monocytes and the dendritic cell subpopulation as well as possible contaminant T lymphocytes. It is based upon the fact that DCs do not express the following 25 markers: CD3 (T cell); CD14 (monocyte); CD16, 56, 57 (NK/LAK cells); CD19, 20 (B cells). At the same time, DCs do express large quantities of HLA-DR, significant HLA-DQ and B7.2 (but little or no B7.1) at the time they are circulating in the blood (in addition they express Leu M7 and M9, myeloid markers which are also expressed by monocytes and neutrophils).

When combined with a third color reagent for analysis of dead cells, propidium iodide (PI), it is possible to make positive identification of all cell subpopulations (see Table 1):

**TABLE 1**  
FACS analysis of fresh peripheral cell subpopulations

|                      | <u>Color #1</u>                        | <u>Color #2</u> | <u>Color #3</u> |
|----------------------|--|-----------------|-----------------|
|                      | <u>Cocktail</u><br>3/14/16/19/20/56/57 | <u>HLA-DR</u>   | <u>PI</u>       |
| Live Dendritic cells | Negative                               | Positive        | Negative        |
| Live Monocytes       | Positive                               | Positive        | Negative        |
| Live Neutrophils     | Negative                               | Negative        | Negative        |
| Dead Cells           | Variable                               | Variable        | Positive        |

Additional markers can be substituted for additional analysis:

Color #1: CD3 alone, CD14 alone, etc.; Leu M7 or Leu M9; anti-Class I, etc.

Color #2: HLA-Dq, B7.1, B7.2, CD25 (IL2r), ICAM, LFA-3, etc.

5

The goal of FACS analysis at the time of collection is to confirm that the DCs are enriched in the expected fractions, to monitor neutrophil contamination, and to make sure that appropriate markers are expressed. This rapid bulk collection of enriched DCs from human peripheral blood, suitable for clinical applications, is absolutely dependent on the analytic FACS technique described above for quality control. If need be, mature DCs can be immediately separated from monocytes at this point by fluorescent sorting for "cocktail negative" cells. It may not be necessary to routinely separate DCs from monocytes because, as will be detailed below, the monocytes themselves are still capable of differentiating into DCs or functional DC-like cells in culture.

20 Specific combination(s) of cytokines have been used successfully to amplify (or partially substitute) for the activation/conversion achieved with calcium ionophore: these cytokines include but are not limited to rhGM-CSF, rhIL-2, and rhIL-4. Each cytokine when given alone is inadequate for optimal upregulation.

In one embodiment, the APCs and cells expressing one or more antigens are autologous. In another embodiment, the APCs and cells expressing the antigen are allogeneic, *i.e.*, derived from a different subject.

5 The following is a brief description of methods to identify and characterize novel tumor antigen for use in the subject invention.

### **Identification of Tumor Associated Antigens**

10 Any conventional method, e.g., subtractive library, comparative Northern and/or Western blot analysis of normal and tumor cells, SAGE (U.S. Patent No. 5,695,937) and SPHERE (described in PCT WO 97/35035), can be used to identify putative antigens for use in the subject invention.

15 SAGE analysis can be employed to identify the antigens recognized by expanded immune effector cells such as CTLs. SAGE analysis involves identifying nucleotide sequences expressed in the antigen-expressing cells. Briefly, SAGE analysis begins with providing complementary deoxyribonucleic acid (cDNA) from (1) the antigen-expressing population and (2) cells not expressing that antigen. Both cDNAs can be linked to primer sites. Sequence tags are then created, for example, using the appropriate primers to amplify the 20 DNA. By measuring the differences in these tags between the two cell types, sequences which are over expressed in the antigen-expressing cell population can be identified.

### **Altered Antigens**

25 Muteins of the antigen as well as allogeneic and antigens from a different species, of previously characterized antigens are useful in the subject invention. Examples of known antigens from which muteins and altered antigens can be derived include, but are not limited to MART1 and gp100. The full-length open reading frame of the mouse MART1 consists of 342 bp, encoding a protein of 113

amino acid residues with a predicted molecular weight of ~13 kDa. Alignment of human and murine MART1 amino acid sequences showed 68.6% identity.

The murine homologue of gp100 has also been identified. The open reading frame consists of 1,878 bp, predicting a protein of 626 amino acid residues which exhibits 75.5% identity to human gp100.

SPHERE, described in PCT WO 97/35035, is a method that will identify wild-type or native antigens as well as provide altered antigens. SPHERE is an empirical screening method for the identification of MHC Class I-restricted CTL epitopes that utilizes peptide libraries synthesized on a solid support (*e.g.*, plastic beads) where each bead contains approximately 200 picomoles of a unique peptide that can be released in a controlled manner. The synthetic peptide library is tailored to a particular HLA restriction by fixing anchor residues that confer high-affinity binding to a particular HLA allele (*e.g.*, HLA-A2) but contain a variable TCR epitope repertoire by randomizing the remaining positions.

Roughly speaking, 50 96-well plates with 10,000 beads per well will accommodate a library with a complexity of approximately  $5 \times 10^7$ . In order to minimize both the number of CTL cells required per screen and the amount of manual manipulations, the eluted peptides can be further pooled to yield wells with any desired complexity. Based on experiments with soluble libraries, it should be possible to screen  $10^7$  peptides in 96-well plates (10,000 peptides per well) with as few as  $2 \times 10^6$  CTL cells. After cleaving a percentage of the peptides from the beads and incubating them with  $^{51}\text{Cr}$ -labeled APCs (*e.g.*, T2 cells) and the CTL line(s), peptide pools containing reactive species can be determined by measuring  $^{51}\text{Cr}$ -release according to standard methods known in the art. Alternatively, cytokine production (*e.g.*, interferon- $\gamma$ ) or proliferation (*e.g.*, incorporation of  $^3\text{H}$ -thymidine) assays may be used. After identifying reactive 10,000-peptide mixtures, the beads corresponding to those mixtures are separated into smaller pools and distributed to new 96-well plates (*e.g.*, 100 beads per well). An additional percentage of peptide is released from each pool and reassayed for activity by one of the methods listed above. Upon identification of

reactive 100-peptide pools, the beads corresponding those peptide mixtures are redistributed at 1 bead per well of a new 96-well plate. Once again, an additional percentage of peptide is released and assayed for reactivity in order to isolate the single beads containing the reactive library peptides. The sequence of the 5 peptides on individual beads can be determined by sequencing residual peptide bound to the beads by, for example, N-terminal Edman degradation or other analytical techniques known to those of skill in the art.

10 *In vitro* confirmation of the immunogenicity of an putative antigen of this invention can be confirmed using the method described below which assays for the generation of CTLs.

### **Presentation of Antigen to the APC**

15 Peptide fragments from antigens must first be bound to peptide binding receptors ((MHC) class I and class II molecules) that display the antigenic peptides on the surface of the APCs. Palmer E. and Cresswell (1998) *Annu. Rev. Immunol.* **16**:323 and Germain R.N. (1996) *Immunol. Rev.* **151**:5. T lymphocytes produce an antigen receptor that they use to monitor the surface of APCs for the presence of foreign peptides. The antigen receptors on T<sub>H</sub> cells recognize 20 antigenic peptides bound to MHC class II molecules whereas the receptors on CTLs react with antigens displayed on class I molecules. For a general review of the methods for presentation of exogenous antigen by APC, see Raychaudhuri and Rock (1998) *Nature Biotechnology* **16**:1025.

25 For purposes of immunization, antigens can be delivered to antigen-presenting cells as protein/peptide or in the form of polynucleotides encoding the protein/peptide *ex vivo* or *in vivo*. The methods described below focus primarily on DCs which are the most potent, preferred APCs.

30 Several different techniques have been described to produce genetically modified APCs. These include: (1) the introduction into the APCs of polynucleotides that express antigen or fragments thereof; (2) infection of APCs with recombinant vectors to induce endogenous expression of antigen; and (3)

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introduction of tumor antigen into the DC cytosol using liposomes. (See Boczkowski D. et al. (1996) *J. Exp. Med.* **184**:465; Rouse et al. (1994) *J. Virol.* **68**:5685; and Nair et al. (1992) *J. Exp. Med.* **175**:609). For the purpose of this invention, any method which allows for the introduction and expression of the heterologous, altered or non-self antigen and presentation by the MHC on the surface of the APC is within the scope of this invention.

### **Antigen Pulsing**

10 Pulsing is accomplished *in vitro/ex vivo* by exposing APCs to antigenic protein or peptide(s). The protein or peptide(s) are added to APCs at a concentration of 1-10  $\mu$ M for approximately 3 hours. Paglia et al. (1996) *J. Exp. Med.* **183**:317, has shown that APC incubated with whole protein *in vitro* were recognized by MHC class I-restricted CTLs, and that immunization of animals with these APCs led to the development of antigen-specific CTLs *in vivo*.

15 Protein/peptide antigen can also be delivered to APC *in vivo* and presented by the APC. Antigen is preferably delivered with adjuvant via the intravenous, subcutaneous, intranasal, intramuscular or intraperitoneal route of delivery. Grant E.P. and Rock K.L. (1992) *J. Immunol.* **148**:13; Norbury, C. C. et al. (1995) *Immunity* **3**:783; and Reise-Sousa C. and Germain R.N. (1995) *J. Exp. Med.* **182**:841.

20

### **Antigen Painting**

25 Another method which can be used is termed "painting". It has been demonstrated that glycosyl-phosphatidylinositol (GPI)-modified proteins possess the ability to reincorporate themselves back into cell membranes after purification. Hirose et al. (1995) *Methods Enzymol.* **250**:582; Medof et al. (1984) *J. Exp. Med.* **160**:1558; Medof (1996) *FASEB J.* **10**:574; and Huang et al. (1994) *Immunity* **1**:607, have exploited this property in order to create APCs of specific composition for the presentation of antigen to CTLs. Expression vectors for  $\beta$ 2-microglobulin and the HLA-A2.1 allele were first devised. The proteins were

30

expressed in Schneider S2 *Drosophila melanogaster* cells, known to support GPI-modification. After purification, the proteins could be incubated together with a purified antigenic peptide which resulted in a trimolecular complex capable of efficiently inserting itself into the membranes of autologous cells. In essence, 5 these protein mixtures were used to “paint” the APC surface, conferring the ability to stimulate a CTL clone that was specific for the antigenic peptide. Cell coating was shown to occur rapidly and to be protein concentration dependent. This method of generating APCs bypasses the need for gene transfer into the APC and permits control of antigenic peptide densities at the cell surfaces.

10

### **Foster Antigen Presenting Cells**

Foster APCs are derived from the human cell line 174xCEM.T2, referred to as T2, which contains a mutation in its antigen processing pathway that restricts the association of endogenous peptides with cell surface MHC class I molecules 15 (Zweerink et al. (1993) *J. Immunol.* **150**:1763). This is due to a large homozygous deletion in the MHC class II region encompassing the genes TAP1, TAP2, LMP1, and LMP2, which are required for antigen presentation to MHC class 1-restricted CD8<sup>+</sup> CTLs. In effect, only “empty” MHC class I molecules are presented on the surface of these cells. Exogenous peptide added to the culture 20 medium binds to these MHC molecules provided that the peptide contains the allele-specific binding motif. These T2 cells are referred to herein as “foster” APCs. They can be used in conjunction with this invention to present the heterologous, altered or control antigen.

Transduction of T2 cells with specific recombinant MHC alleles allows for 25 redirection of the MHC restriction profile. Libraries tailored to the recombinant allele will be preferentially presented by them because the anchor residues will prevent efficient binding to the endogenous allele.

High level expression of MHC molecules makes the APC more visible to the CTLs. Expressing the MHC allele of interest in T2 cells using a powerful 30 transcriptional promoter (e.g., the CMV promoter) results in a more reactive APC

(most likely due to a higher concentration of reactive MHC-peptide complexes on the cell surface).

### Hybrid APCs

5 WO 98/58541 describes a method to fuse cells expressing an antigen with dendritic cells in a manner that the dendritic cells take up and present the antigens expressed by the antigen-expressing cells. The DCs are fused with the cells in the presence of a fusing agent (e.g., polyethylene glycol or Sendai virus). After culturing the post fusion cell mixture in a medium (which optionally contains 10 hypoxanthine, aminopterin and thymidine) for a period of time (e.g., 5-12 days), the cultured fused cells are separated from unfused non-DC parental cells based on the different adherence properties of the two cell groups. The unfused parental DCs do not proliferate, and so die off.

### Production of Epitope or Antigen

15 Most preferably, isolated peptides of the present invention can be synthesized using an appropriate solid state synthetic procedure. Steward and Young, Solid Phase Peptide Synthesis, Freemantle, San Francisco, Calif. (1968). A preferred method is the Merrifield process. Merrifield, Recent Progress in 20 Hormone Res., 23:451 (1967). The antigenic activity of these peptides may conveniently be tested using, for example, the assays as described herein.

Once an isolated peptide of the invention is obtained, it may be purified by standard methods including chromatography (e.g., ion exchange, affinity, and sizing column chromatography), centrifugation, differential solubility, or by any 25 other standard technique for protein purification. For immunoaffinity chromatography, an epitope may be isolated by binding it to an affinity column comprising antibodies that were raised against that peptide, or a related peptide of the invention, and were affixed to a stationary support.

30 Alternatively, affinity tags such as hexa-His (Invitrogen), Maltose binding domain (New England Biolabs), influenza coat sequence (Kolodziej et al. (1991)

Methods Enzymol. 194:508-509), and glutathione-S-transferase can be attached to the peptides of the invention to allow easy purification by passage over an appropriate affinity column. A DNA affinity column using DNA containing a sequence encoding the peptides of the invention could be used in purification.

5 Isolated peptides can also be physically characterized using such techniques as proteolysis, nuclear magnetic resonance, and x-ray crystallography.

Another aspect of the invention is isolated nucleic acid sequences that encode the novel antigenic peptides described herein. With regard to nucleic acid sequences of the present invention, "isolated" means: an RNA or DNA polymer, portion of genomic nucleic acid, cDNA, or synthetic nucleic acid which, by virtue 10 of its origin or manipulation: (i) is not associated with all of a nucleic acid with which it is associated in nature (e.g. is present in a host cell as a portion of an expression vector); or (ii) is linked to a nucleic acid or other chemical moiety other than that to which it is linked in nature; or (iii) does not occur in nature. By 15 "isolated" it is further meant a nucleic acid sequence: (i) amplified in vitro by, for example, polymerase chain reaction (PCR); (ii) synthesized by, for example, chemical synthesis; (iii) recombinantly produced by cloning; or (iv) purified, as by cleavage and gel separation.

20 The nucleic acid sequences of the present invention may be characterized, isolated, synthesized and purified using no more than ordinary skill. The antigens and fragments thereof may be recombinantly produced and isolated for use in the subject invention using methods well known in the art and described below. See 25 Sambrook et al., (1989) *supra*.

## 25 **Vectors Useful in Genetic Modifications**

In one aspect of the invention, the antigen is delivered to the antigen presenting cell (APC) by a gene delivery vehicle, preferably an adenoviral vector. Thus, the APC is genetically modified to express the antigen. In general, genetic 30 modifications of cells employed in the present invention are accomplished by introducing a vector containing a polypeptide or transgene encoding a

heterologous or an altered antigen. A variety of different gene transfer vectors, including viral as well as non-viral systems can be used. Viral vectors useful in the genetic modifications of this invention include, but are not limited to adenovirus, adeno-associated virus vectors, retroviral vectors and adeno-retroviral chimeric vectors. Preferably, a recombinant adenoviral vector is used to genetically modify the DC cells.

#### **Construction of Recombinant Adenoviral Vectors or Adeno-Associated Virus Vectors, Including Chimeric Adenoviral Vectors**

Adenovirus and adeno-associated virus vectors useful in the genetic modifications of this invention may be produced according to methods already taught in the art. (see, *e.g.*, Karlsson et al. (1986) EMBO 5:2377; Carter (1992) Current Opinion in Biotechnology 3:533-539; Muzyczka (1992) Current Top. Microbiol. Immunol. 158:97-129; GENE TARGETING: A PRACTICAL APPROACH (1992) ed. A. L. Joyner, Oxford University Press, NY). Several different approaches are feasible. Preferred is the helper-independent replication deficient human adenovirus system.

Additional references describing adenovirus vectors and other viral vectors which could be used in the methods of the present invention include the following: Horwitz, M.S., Adenoviridae and Their Replication, in Fields et al. (eds.) VIROLOGY, Vol. 2, Raven Press New York, pp. 1679-1721, 1990); Graham et al., pp. 109-128 in METHODS IN MOLECULAR BIOLOGY, Vol. 7: GENE TRANSFER AND EXPRESSION PROTOCOLS, Murray (ed.), Humana Press, Clifton, N.J. (1991); Miller et al. (1995) FASEB Journal 9:190-199; Schreier (1994) Pharmaceutica Acta Helveticae 68:145-159; Schneider and French (1993) Circulation 88:1937-1942; Curiel et al. (1992) Human Gene Therapy 3:147-154; Graham et al., WO 95/00655; Falck-Pedersen WO 95/16772; Denefle et al., WO 95/23867; Haddada et al., WO 94/26914; Perricaudet et al. WO 95/02697; and Zhang et al. WO 95/25071. A variety of adenovirus plasmids are also available from commercial sources, including, *e.g.*, Microbix Biosystems of Toronto, Ontario (see, *e.g.*, Microbix

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Product Information Sheet: Plasmids for Adenovirus Vector Construction, 1996). See also, the papers by Vile et al. (1997) *Nature Biotechnology* **15**: 840-841 and Feng et al.(1997) *Nature Biotechnology*, **15**: 866-870, describing the construction and use of adeno-retroviral chimeric vectors that can be employed for genetic modifications.

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Also useful in the practice of the present invention are chimeric adenoviral vectors, for example as described in PCT/US97/21494, the disclosure of which is hereby incorporated by reference. A "chimeric adenoviral vector" is an adenoviral vector that comprises polynucleotides from more than one adenovirus serotype.

15

Briefly, the chimeric vectors contain an adenoviral backbone, for example, Ad 2/CFTR-1 and Ad 2 FTR2 and others described in U. S. Patent No. 5,670,488, issued September 23, 1997. Such vectors may include deletion of the E1 region, partial or complete deletion of the E4 region, and deletions within, for example, the E2 and E3 regions.

Construction of the chimeric adenoviral vectors can be based on adenovirus DNA sequence information widely available in the field, e.g., nucleic acid sequence databases such as GenBank.

20

Preparation of replication-defective chimeric adenoviral vector stocks can be accomplished using cell lines that complement viral genes deleted from the vector, e.g., 293 or A549 (available from the ATCC) cells containing the deleted adenovirus E1 genomic sequences. The use of HER3 cells (human embryonic retinoblasts transformed by Ad 12), as a complementing cell line is of note. After amplification of plaques in suitable complementing cell lines, the viruses can be recovered by freeze-thawing and subsequently purified using cesium chloride centrifugation. Alternatively, virus purification can be performed using chromatographic techniques, e.g., as set forth in International Application No. PCT/US96/13872, filed August 30, 1996.

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Titers of replication-defective chimeric adenoviral vector stocks can be determined by plaque formation in a complementing cell line, e.g., 293 cells.

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Endpoint dilution using an antibody to the adenoviral hexon protein may be used to quantitate virus production or infection efficiency of target cells (Armentano et al. (1995) *Hum. Gene Ther.* 6:1343-53.

In addition to containing the DNA sequences encoding one or more transgenes, the chimeric adenoviral vectors of the invention may contain any expression control sequences such as a promoter or enhancer, a polyadenylation element, and any other regulatory elements that may be used to modulate or increase expression, all of which are operably linked in order to allow expression of the transgene. The use of any expression control sequences, or regulatory elements, which facilitate expression of the transgene is within the scope of the invention. Such sequences or elements may be capable of generating tissue-specific expression or be susceptible to induction by exogenous agents or stimuli.

### **Construction of Retroviral Vectors**

The antigen presenting cells described herein can also be genetically modified with retroviral vectors produced recombinantly by procedures already taught in the art. For example, WO 94/29438 describes the construction of retroviral packaging plasmids and packaging cell lines. As is apparent to the skilled artisan, the retroviral vectors useful in the methods of this invention are capable of infecting the cells described herein. The techniques used to construct vectors, and transfet and infect cells are widely practiced in the art. Examples of retroviral vectors are those derived from murine, avian or primate retroviruses. Retroviral vectors based on the Moloney murine leukemia virus (MoMLV) are the most commonly used because of the availability of retroviral variants that efficiently infect human cells. Other suitable vectors include those based on the Gibbon Ape Leukemia Virus (GALV) or HIV.

The range of host cells that may be infected by a retrovirus or retroviral vector is determined by the viral envelope protein. The recombinant virus can be used to infect virtually any other cell type recognized by the env protein provided by the packaging cell, resulting in the integration of the viral genome in the

transduced cell and the stable production of the foreign gene product. In general, murine ecotropic env of MoMLV allows infection of rodent cells, whereas amphotropic env allows infection of rodent, avian and some primate cells, including human cells. Amphotropic packaging cell lines for use with MoMLV systems are known in the art and commercially available and include, but are not limited to, PA12 and PA317. Miller et al. (1985) Mol. Cell. Biol. **5**:431-437; Miller et al. (1986) Mol. Cell. Biol. **6**:2895-2902; and Danos et al. (1988) Proc. Natl. Acad. Sci. USA **85**:6460-6464. Xenotropic vector systems exist which also allow infection of human cells.

The host range of retroviral vectors has been altered by substituting the env protein of the base virus with that of a second virus. The resulting, "pseudotyped", virus has the host range of the virus donating the envelope protein and expressed by the packaging cell line. Recently, the G-glycoprotein from vesicular stomatitis virus (VSV-G) has been substituted for the MoMLV env protein. Burns et al. (1993) Proc. Natl. Acad. Sci USA **90**:8033-8037; and PCT patent application WO 92/14829. Since infection is not dependent on a specific receptor, VSV-G pseudotyped vectors have a broad host range.

Usually, the vectors will contain at least two heterologous genes or gene sequences: (i) the therapeutic gene to be transferred; and (ii) a marker gene that enables tracking of infected cells.

The viral vector can be modified to incorporate chimeric envelope proteins or nonviral membrane proteins into retroviral particles to improve particle stability and expand the host range or to permit cell type-specific targeting during infection. The production of retroviral vectors that have altered host range is taught, for example, in WO 92/14829 and WO 93/14188. Retroviral vectors that can target specific cell types *in vivo* are also taught, for example, in Kasahara et al. (1994) Science **266**:1373-1376. Kasahara et al. describe the construction of a Moloney leukemia virus (MoMLV) having a chimeric envelope protein consisting of human erythropoietin (EPO) fused with the viral envelope protein. This hybrid virus shows tissue tropism for human red blood progenitor cells that bear the

receptor for EPO, and is therefore useful in gene therapy of sickle cell anemia and thalassemia. Retroviral vectors capable of specifically targeting infection of cells are preferred for *in vivo* gene therapy.

The vector construct is introduced into a packaging cell line which will generate infectious virions. Packaging cell lines capable of generating high titers of replication-defective recombinant viruses are known in the art, see for example, WO 94/29438. Viral particles are harvested from the cell supernatant and purified for *in vivo* infection using methods known in the art such as by filtration of supernatants 48 hours post transfection. The viral titer is determined by infection of a constant number of appropriate cells (depending on the retrovirus) with titrations of viral supernatants. The transduction efficiency can be assayed 48 hours later by a variety of methods, including Southern blotting.

After viral transduction, the presence of the viral vector in the transduced cells or their progeny can be verified such as by PCR. PCR can be performed to detect the marker gene or other virally transduced sequences. Generally, periodic blood samples are taken and PCR conveniently performed using e.g. NeoR probes if the NeoR gene is used as marker. The presence of virally transduced sequences in bone marrow cells or mature hematopoietic cells is evidence of successful reconstitution by the transduced cells. PCR techniques and reagents are well known in the art, See, generally, PCR PROTOCOLS, A GUIDE TO METHODS AND APPLICATIONS. Innis, Gelfand, Sninsky & White, eds. (Academic Press, Inc., San Diego, 1990) and commercially available (Perkin-Elmer).

*In vitro/ex vivo*, exposure of human DCs to adenovirus (Ad) vector at a multiplicity of infection (MOI) of 500 for 16-24 h in a minimal volume of serum-free medium reliably gives rise to transgene expression in 90-100% of DCs. The efficiency of transduction of DCs or other APCs can be assessed by immunofluorescence using fluorescent antibodies specific for the tumor antigen being expressed (Kim et al., 1997). Alternatively, the antibodies can be conjugated to an enzyme (e.g. HRP) giving rise to a colored product upon reaction

with the substrate. The actual amount of antigen being expressed by the APCs can be evaluated by ELISA.

Transduced APCs can subsequently be administered to the host via an intravenous, subcutaneous, intranasal, intramuscular or intraperitoneal route of delivery.

*In vivo* transduction of DCs, or other APCs, can potentially be accomplished by administration of Ad (or other viral vectors) via different routes including intravenous, intramuscular, intranasal, intraperitoneal or cutaneous delivery. The preferred method is cutaneous delivery of Ad vector at multiple sites using a total dose of approximately  $1 \times 10^{10}$ - $1 \times 10^{12}$  i.u. Levels of *in vivo* transduction can be roughly assessed by co-staining with antibodies directed against APC marker(s) and the TAA being expressed. The staining procedure can be carried out on biopsy samples from the site of administration or on cells from draining lymph nodes or other organs where APCs (in particular DCs) may have migrated. The amount of TAA being expressed at the site of injection or in other organs where transduced APCs may have migrated can be evaluated by ELISA on tissue homogenates.

Although viral gene delivery is more efficient, DCs can also be transduced *in vitro/ex vivo* by non-viral gene delivery methods such as electroporation, calcium phosphate precipitation or cationic lipid/plasmid DNA complexes. Transduced APCs can subsequently be administered to the host via an intravenous, subcutaneous, intranasal, intramuscular or intraperitoneal route of delivery.

*In vivo* transduction of DCs, or other APCs, can potentially be accomplished by administration of cationic lipid/plasmid DNA complexes delivered via the intravenous, intramuscular, intranasal, intraperitoneal or cutaneous route of administration. Gene gun delivery or injection of naked plasmid DNA into the skin also leads to transduction of DCs. Intramuscular delivery of plasmid DNA may also be used for immunization.

The transduction efficiency and levels of transgene expression can be assessed as described above for viral vectors.

### Expansion of Immune Effector Cells

5 The present invention makes use of these APCs to stimulate production of an enriched population of antigen-specific immune effector cells. The antigen-specific immune effector cells are expanded at the expense of the APCs, which die in the culture. The process by which naïve immune effector cells become educated by other cells is described essentially in Coulie (1997) *Molec. Med.*

10 Today 3:261-268.

In a preferred embodiment, the antigen-specific immune effector cells are CTLs. In one aspect, the cytotoxic T cells are polyclonal T cells isolated from a site of cytotoxic T cell infiltration from a subject. Alternatively, such cells may be isolated from a site of cytotoxic T cell infiltration from two or more subjects or 15 human patients, in which the subjects share an MHC haplotype. In another embodiment, the CTLs may be two or more cytotoxic T cell lines. In yet another embodiment, the CTLs may be any combination of the foregoing.

20 In a further aspect of the invention, the site of cytotoxic T cell infiltration is a tumor. The tumors from which cells or cell lines are obtained can be the same type of tumor in different individuals with a shared MHC haplotype or different types of tumors from different subjects who share an MHC haplotype.

25 The APCs prepared as described above are mixed with naïve immune effector cells. Preferably, the cells may be cultured in the presence of a cytokine, for example IL2. Because dendritic cells secrete potent immunostimulatory cytokines, such as IL2, it may not be necessary to add supplemental cytokines during the first and successive rounds of expansion. In any event, the culture conditions are such that the antigen-specific immune effector cells expand (*i.e.* proliferate) at a much higher rate than the APCs. Multiple infusions of APCs and optional cytokines can be performed to further expand the population of antigen-specific cells.

In one embodiment, the immune effector cells are T cells and are specific for tumor-specific antigens which are presented by the APCs.

### Compositions

5 This invention also provides compositions containing any of the above-mentioned proteins, muteins, polypeptides, nucleic acid molecules, vectors, cells antibodies and fragments thereof, and an acceptable solid or liquid carrier. When the compositions are used pharmaceutically, they are combined with a "pharmaceutically acceptable carrier" for diagnostic and therapeutic use. These 10 compositions also can be used for the preparation of medicaments for the diagnosis and treatment of diseases such as cancer.

### Tumor Protection in Animal Models

15 Applicants are the first to establish that, based on the animal models described below, prevaccination with the compositions of this invention will prevent or delay onset of disease.

20 The murine B16 melanoma model was used. In this model, C57BL/6 mice were immunized with bone marrow-derived DCs transduced with an Ad vector encoding either human gp100 (Ad/hugp100) or mouse gp100 (Ad/mgpl00). Mice immunized against heterologous human gp100 developed a protective immune response and were resistant to a lethal subcutaneous challenge of B16 melanoma cells (syngeneic tumor cell line that expresses gp100). In contrast, mice 25 immunized with homologous mouse gp100 failed to mount a protective immune response against B16 melanoma cells and developed tumors at the site of B16 cell injection. This finding illustrates the difficulty in breaking tolerance against a self antigen (mouse gp100). The corresponding heterologous antigen from a different species (human gp100), however, is likely to contain several Class I and Class II-associated epitopes that will be recognized as foreign and elicit CD8<sup>+</sup> and CD4<sup>+</sup> T cell responses, respectively. The induction of cross-reactive CTLs that recognize

both the heterologous and homologous self-antigen can then lead to lysis of host tumor cells.

Unfortunately, this type of animal model cannot be used to test the efficacy of modified or heterologous tumor antigens being considered for use in humans since mice and humans recognize different epitopes, primarily as a result of differences in their MHC molecules. It may be possible, however, to use the allogeneic human peripheral blood lymphocyte - severe combined immunodeficiency mouse (Hu-PBL-SCID) model. SCID mice lack mature B and T lymphocytes and can be reconstituted with human PBLs. It may be possible to immunize such mice with test antigen to induce a response in adoptively transferred human PBLs and evaluate protection against challenge with a human tumor cell line (Mosier et al., 1988; Parney et al., 1997; Albert et al., 1997).

Another possibility is immunization of HLA-A2.1 transgenic mice to reproduce the immune reactivity of HLA-A2 individuals.

### **Adoptive Immunotherapy and Vaccines**

The expanded populations of antigen-specific immune effector cells of the present invention find use in adoptive immunotherapy regimes and as vaccines.

Adoptive immunotherapy methods involve, in one aspect, administering to a subject a substantially pure population of educated, antigen-specific immune effector cells made by culturing naïve immune effector cells with APCs as described above. Preferably, the APCs are dendritic cells.

In one embodiment, the adoptive immunotherapy methods described herein are autologous. In this case, the APCs are made using parental cells isolated from a single subject. The expanded population also employs T cells isolated from that subject. Finally, the expanded population of antigen-specific cells is administered to the same patient.

In another embodiment, the adoptive immunotherapy methods are allogeneic. Here, cells from two or more patients are used to generate the APCs, and stimulate production of the immune effector cells. For instance, cells from

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other healthy or diseased subjects can be used to generate antigen-specific cells in instances where it is not possible to obtain autologous T cells and/or dendritic cells from the subject providing the biopsy. The expanded population can be administered to any one of the subjects from whom cells were isolated, or to another subject entirely.

In a further embodiment, APCs or immune effector cells are administered with an effective amount of a stimulatory cytokine, such as IL-2 or a co-stimulatory molecule.

10 The agents identified herein as effective for their intended purpose can be administered to subjects or individuals susceptible to or at risk of developing a disease, such as cancer. When the agent is administered to a subject such as a mouse, a rat or a human patient, the agent can be added to a pharmaceutically acceptable carrier and systemically or topically administered to the subject. To determine patients that can be beneficially treated, a tumor regression can be assayed. Therapeutic amounts can be empirically determined and will vary with the pathology being treated, the subject being treated and the efficacy and toxicity of the therapy. When delivered to an animal, the method is useful to further confirm efficacy of the agent. As an example of an animal model, groups of nude mice (Balb/c NCR nu/nu female, Simonsen, Gilroy, CA) are each subcutaneously inoculated with about  $10^5$  to about  $10^9$  hyperproliferative, cancer or target cells as defined herein. When the tumor is established, the agent is administered, for example, by subcutaneous injection around the tumor. Tumor measurements to determine reduction of tumor size are made in two dimensions using venier calipers twice a week. Other animal models may also be employed as appropriate.

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Administration *in vivo* can be effected in one dose, continuously or intermittently throughout the course of treatment. Methods of determining the most effective means and dosage of administration are well known to those of skill in the art and will vary with the composition used for therapy, the purpose of the therapy, the target cell being treated, and the subject being treated. Single or

multiple administrations can be carried out with the dose level and pattern being selected by the treating physician. Suitable dosage formulations and methods of administering the agents can be found below.

5 The agents and compositions of the present invention can be used in the manufacture of medicaments and for the treatment of humans and other animals by administration in accordance with conventional procedures, such as an active ingredient in pharmaceutical compositions.

10 More particularly, an agent of the present invention also referred to herein as the active ingredient, may be administered for therapy by any suitable route including nasal, topical (including transdermal, aerosol, buccal and sublingual), parenteral (including subcutaneous, intramuscular, intravenous and intradermal) and pulmonary. It will also be appreciated that the preferred route will vary with the condition and age of the recipient, and the disease being treated.

15 The compositions and methods described herein are particularly useful in providing or inducing a prophylactic immune response in an animal. Animals in a pre-disease state or in a disease free interval, i.e., having or pre-disposed to a condition subject to immune surveillance, are most suitably treated by the methods and compositions described herein. Such conditions involve the activation of an immune response in a diseased state or period.

20 **Example**

25 Dendritic cells were derived from peripheral blood of a HLA-A2+ human donor using standard GM-CSF/IL-4 culture technique. After six days, the cells were infected with an adenovirus (serotype 2) construct encoding human gp 100. At T=7 days, they were restimulated with  $5 \times 10^5$  infected autologous DCs (from frozen stocks) and given 50 U/ml rhIL-2. At T=14 days, the cultures were treated with leucyl-leucyl-methyl ester in order to eliminate NK cell activity. Immediately after treatment, the cells were washed thoroughly and replated along 30 with  $1 \times 10^7$  mitomycin C-treated autologous PBMC as feeders. At T=21 days,

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cells were split and replated at  $5 \times 10^5$  cells/ml in Iscoves/10% human AB serum/1000U rhIL-2/ml. At T=26 days, the CTLs raised against gp100 were tested in  $^{51}\text{Cr}$ -release assay using peptide pulsed T2 cells as targets. Effector CTLs were TIL 1520 which specifically recognize an HLA-A-A2 restricted epitope of the wild-type human gp100 protein. Figure 5 shows the results of this assay. There is epitope-specific recognition that was not present prior to education of the T cells with the infected DCs.

10

It is to be understood that while the invention has been described in conjunction with the above embodiments, that the foregoing description and the following examples are intended to illustrate and not limit the scope of the invention. For example, any of the above-noted compositions and/or methods can be combined with known therapies or compositions. Other aspects, advantages and modifications within the scope of the invention will be apparent to those skilled in the art to which the invention pertains.

15

CLAIMS

1. A method of inducing a prophylactic immune response to a self-antigen in a subject, comprising administering to the subject an effective amount of the antigen or an altered form of the antigen.

5 2. The method of claim 1, wherein the antigen is administered as a polynucleotide coding for the self-antigen.

10 3. The method of claim 2, wherein the polynucleotide is delivered as naked DNA.

4. The method of claim 2, wherein the polynucleotide is delivered in a gene delivery vehicle.

15 5. The method of claim 1, further comprising administering an effective amount of an immunostimulatory agent to the subject.

20 6. The method of claim 5, wherein the immunostimulatory agent is administered as a polynucleotide coding for the immunostimulatory agent.

7. The method of claim 1, wherein the antigen is administered in an antigen presenting cell.

25 8. The method of claim 7, wherein the antigen presenting cell has been genetically modified by insertion of a polynucleotide coding for the antigen.

30 9. The method of claim 7, wherein the antigen presenting cell is a foster antigen presenting cell, a hybrid antigen presenting cell, or a pulsed antigen presenting cell.

10. The method of claim 7, wherein the antigen presenting cell is a dendritic cell.

5 11. The method of claim 7, further comprising administering an effective amount of an immunostimulatory agent to the subject.

12. The method of claim 11, wherein the immunostimulatory agent is administered as a polynucleotide coding for the immunostimulatory agent.

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13. The method of claim 1 or 7, wherein the self-antigen is a tumor associated antigen (TAA).

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14. A method of providing a prophylactic immune response to a self-antigen in a subject, comprising administering to the subject an effective amount of educated immune effector cells, educated to specifically recognize and lyse cells expressing the self-antigen or an altered form of the self-antigen.

20

15. The method of claim 14, wherein the immune effector cells have been produced by stimulating naïve immune effector cells with antigen presenting cells that present the antigen or an altered self-antigen to the naïve immune effector cells.

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16. The method of claim 14, wherein the educated immune effector cells are produced *ex vivo*.

17. The method of claim 14, wherein the educated immune effector cells are produced *in vivo*.

18. The method of claim 14, further comprising administering an effective amount of an immunostimulatory agent.

5 19. The method of claim 18, wherein the immunostimulatory agent is administered as a polynucleotide coding for the immunostimulatory agent.

10 20. The method of claim 1 or 14, wherein the subject is characterized as being in a disease-free state but genetically predisposed to a condition subject to immune surveillance.

21. The method of claim 20, wherein the condition is associated with the presence of the HER-2/neu gene in the subject.

15 22. The method of claim 1 or 14, wherein the subject is characterized as being in a disease free interval of a condition subject to immune surveillance.

23. The method of claim 22, wherein the condition is melanoma.

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FIG. 1A

FIG. 1B

FIG. 1

|       |     |             |              |              |               |                |
|-------|-----|-------------|--------------|--------------|---------------|----------------|
| HUMAN | 1   | MDLVLKRCLL  | HLAVIGALLA   | VGATKVPRNQ   | DWLGVSRQLR    | TKAWNRQLYP     |
| MOUSE |     | -★-QR-SF-   | PLV-LSA---   | ---LEGS---   | ---P---V      | --T---         |
|       |     |             |              |              |               |                |
|       | 51  | EWTEAQRLDC  | WRGGQVSLKV   | SNDGPTLIGA   | NASF\$IALNF   | PGSQKVLPDG     |
|       |     | ---V-GSN-   | -----R-      | ----- ---    | -----H-       | -----          |
|       |     |             |              |              |               |                |
|       | 101 | QVIWVNNTII  | NGSQVWWGGQP  | VYPQETDDAC   | IFPDGGPCPS    | GWSQKRSFV      |
|       |     | ---A---     | -----        | -----P-----  | -----V-----   | -----PKPP----- |
|       |     |             |              |              |               |                |
|       | 151 | YVWKTWGQYW  | QVLGGPVSGL   | SIGTGGRAMLG  | THTMEVTYH     | RRGSRSYVPL     |
|       |     | ---K--      | -----R-      | ---A---H-K-- | -----         | ---Q-----      |
|       |     |             |              |              |               |                |
|       | 201 | AHSSSAFTIT  | DQVPFSVSVS   | QLRALDGGNK   | HFLRNQPLTF    | ALQLHDPSGY     |
|       |     | ---A---T--- | -----        | -----Q-----  | -----ET-      | -----H---I     |
|       |     |             |              |              |               |                |
|       | 251 | LAEADLSYTW  | DFGDSGCTL    | SRALVVTHTY   | LEPGPVTAQV    | VLQAAIPLTS     |
|       |     | -----       | -----GT----- | -----D-----  | -----S-S----- | -----V-----    |

FIG. 1A

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301 CGSSPVPGTT DGHRPTEAEP NTTAGQVPTT EVVGTTPGQA PTAEPSGTT  
 ----- --YM----- G--SR-GT-- ----- M --TQ-----V

351 VQVPTTEVIS TAPVQMPATAE STGMTPEKVP VSEVMGTTLA EMSTPEATGM  
 --M-----TA -TSE--L-\* \*----- \*----- \*----- \*-----

401 TPAEVSVVVL SGTTAAQVTT TEWVETTARE LPipeEGPD ASSIMSTESI  
 --T\*-\*-\*P -----V--A-- ---\*----- \*----- \*----- --PLLP-Q-S

451 TGGLGPLLDG TATLRLVKRQ VPLDCVLYRY GSFSVTLDIV QGIESAEILQ  
 ---S----D -D-IM----- -----LA-----

501 AVPSGEGDAF ELTVSCQGGL PKEACMEISS PGCQPPAQRL CQPVLPSPAC  
 ---FS----- -----D----- -----S-----D-----

551 QLVLHQILKG GSGTYCLNVS LADTNSLAVV STQLIMPQQE AGLGQVPLIV  
 -----V--- -----A-----A-----VV---D G-----A--L-

601 GILLVLMAVV LASLIYRRRL MKQDFSVPQL PHSSSHWLRL PRIFCSCPIG  
 -----V--- -----H--K--G\*--S--M --G-T----- -PV-RARGL-

651 ENSPLLSGQQ VX

FIG. 1B

FIG. 2A

4

FIG. 2A

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FIG. 2B

FIG. 2

|       |   |    |
|-------|---|----|
| mMART | ATGCCCAAGAACATTCACTT-----TGTTATCCCAGGAAGGGCACAGACGCTCC  | 50 |
| hMART | ATCCAAGAGATGCTCACTCATCTATGGTTACCCAAAGAACGGGCACGGCCACTCT | 50 |

|       |   |     |     |     |     |     |
|-------|---|-----|-----|-----|-----|-----|
| mMART | 120   | 130 | 140 | 150 | 160 | 170 |
|       | CTGCTTATCGGCTGGTACTGTAGAACGAAAGTGATACAGAACCTTGATGGACAAA |     |     |     |     |     |
|       |   |     |     |     |     |     |
| hMART | 130   | 140 | 150 | 160 | 170 | 180 |
|       | CTGCTCATCGGCTGGTATTGTAGAACGAAATGGATACAGGCCTTGATGGATAAA  |     |     |     |     |     |
|       |   |     |     |     |     |     |

|       |          |             |             |              |            |         |
|-------|----------|-------------|-------------|--------------|------------|---------|
| mmART | 180      | 190         | 200         | 210          | 220        | 230     |
|       | AGGGTCA  | TATTGATTCAA | AAACCTCAAGG | AAAGATGCTCA  | TGTGAGGCC  | CTGAT   |
|       |          |             |             |              |            |         |
| hMART | 190      | 200         | 210         | 220          | 230        | 240     |
|       | AGTCTCAT | GTTGCCACTCA | ATGTGCCCTA  | ACAAGAAGTCCC | ACACAAGAAG | GGTTGAT |
|       |          |             |             |              |            |         |

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| B   | mMART   | hMART  | mMART | hMART |
|-----|---|--|-------|-------|
|     | MPQEDIHF--GYPRKGHRRSYVTAAEAAAGIGILIVVLGIALLIGCWYCRRRSGYRTLMDK | MPREDAHF1YGYPKKGHHSYTTAAEAAAGIGILTVILGVLLIGCWYCRRRNGYRALMDK  |       |       |
| 10  |   |  | 10    |       |
| 20  |   |  | 20    |       |
| 30  |   |  | 30    |       |
| 40  |   |  | 40    |       |
| 50  |   |  | 50    |       |
|     |   |  |       |       |
| 60  | RRHIGIQLKTSRERCSCESPDHQDSRLSSQEKSHPVVPNAPPAYEKLSS--SPPPYSPX   | SLHVGTQCALTRRCPQEGFDHRRDSKVSLQEKNCPEVVPNAPPAYEKLSAEQSPPPYSPX |       |       |
| 70  |   |  | 70    |       |
| 80  |   |  | 80    |       |
| 90  |   |  | 90    |       |
| 100 |   |  | 100   |       |
| 110 |   |  | 110   |       |

**RECTIFIED SHEET (RULE 91)**

FIG. 2B

FIG. 3A    FIG. 3B    } FIG. 3

1 AGCAGACAGGGACTCTCATTAAGGAAGG    TGTCTCTGTGCCCTTGACCCCTACAAGATGCCA  
Met Pro

120 ACGGCTGAAGAGGCCGCTGGGATCGGCATC    CTGACAGTGATCCTGGGACTCTTACTTGCTC  
23    ThrAlaGluGluAlaAlaGlyIleGlyIle LeuThrValIleLeuGlyValLeuLeu

240 CATGTTGGCACTCAATGTGCCCTAACAAAGA    AGATGCCCACAAAGAAGGGTTGATCATCGG  
63    HisValAlaGlyThrGlyInCysAlaLeuThrArg ArgCysProGlnGluGlyPheAspHisArg

360 GCTTATGAGAAACTCTCTGCAGAACAGTCA    CCACACCTTATTCCACCTTAAGAGCCAGCG  
103    AlaTyrGluIleSerAlaGluGlnSer ProProProTyrSerPro

480 ATCTAATGTTCTCCTTGGAAATGGTTAGG    AAAAATGCCAAGCCATCTCTATAATAAGTC  
500 TATTAATTGGAAAACCTCCATCAATAAT    GTTGCATGCAATGATACTATCTGTGCCAGA  
520 GGGCCATCCAATTCTCTTACTTGAAT    TTGGCTAATAACAAACTAGCAGTTTCG  
540 GATACCTTACAGGTTAACACAAGGGTTG    ACTGGCCTATTATCTGATCAAGAACATGT  
560 CTATAGCTCTTTTTGAGATGGAGTTT    CGCTTTGTTGCCAGGCTGAGTGCAATG  
580 CCTCCTGAGTAGCTGGATTACAGGGTGC    GCCACTATGCCGTGACTAATTGTAGTTT  
600 TCTGCCGCTCAGCTCCCAAAGTGGGG    AATTACAGGGTGAGCCACCACGGCTGGCT  
620 AATGCTTACTTAACATAATGACAAGTATTT    CTACTAAACAGAAATTGGTAGAAGGATT  
640 TACCTATGCCAATTAGCTCTTGGGTTT    CCAAATCCCTCTCACAAAGAATGTGCCAGAAG

FIG. 3A

|                                    |  |            |
|------------------------------------|--|------------|
| AGAGAAGATGCTCACTTCATCTATGGTTAC     | CCCAAGAAGGGGCACGGCCACTCTTACACC         | 119        |
| ArgGluAspAlaHisPheIleTyrglyTyr     | ProLysGlyHisGlyHisSerTyrThr            | 22         |
| <u>IleGlyCystrpTyrCysArgArgAsn</u> | <u>GGATACAGAGCCCTTGATGGATAAAAGTCTT</u> | <u>239</u> |
| AspSerLysValSerLeuGlnGlyLysAsn     | GlyTyrArgAlaLeuMetAspLysSerLeu         | 62         |
| GACAGCAAAGTGTCTCAAGAGAAAAAC        | TGTGAACCTGTGGTTCCCAATGCTCCACCT         | 359        |
| AspSerLysValSerLeuGlnGlyLysAsn     | CysGluProValValProAsnAlaProPro         | 102        |
| AGACACCTGAGACATGCTGAAATTATTCT      | CTCACACCTTTGCTTGAATTAAATACAGAC         | 479        |
|                                    |  | 118        |
| AGTGTTAAATTAGTAGGTCCGCTAGCA        | GTACTAATCATGTTGAGGAATGATGAGAAA         | 599        |
| GGTAATGTTAGAAATCCATGGTTATT         | TCTGAGAGACAGAAATTCAAGTGGGTATTCT        | 719        |
| AACCTTGACCGACATGAACCTGACAGAA       | TTGTTCCAGTACTATGGAGTGCACAAAG           | 839        |
| CAGCAATGTCCTTGTGCTCTAAATTCT        | ATATACTACAAATAATATTGTAAGATC            | 959        |
| GGCGATCTGGCTACCCATAACCTCCGCC       | TCCCAGGGTCAAGCAATTCTCCTGCCTTAG         | 1079       |
| AGTAGAGACGGGGTTCTCATGGTCA          | GGCTGGTCTCAAACCTGACCTCAGGTGA           | 1199       |
| GGATCCTATATCTTAGGTAAAGACATAAC      | GGAGTCTAATTACATTCAACTCAGGCTC           | 1319       |
| AAATAAGTAAAGCTACTATGTACTGCCCT      | AGTGCTGATGCCCTGTGTACTGCCCTAAATG        | 1439       |
| AAATCATAAAGGATCAGAGATTCTGAAAAA     | AAAAAAAAAAAAAAAAAAAAAA                 | 1559       |

FIG. 3B

FIG. 4A

FIG. 4B

## MOUSE TRP2

1 MGLVGWGLLL GCLGGCILLR ARAQFPRVCM TLDGVLNKEC CPPLGPEATN  
 51 ICGFLEGRGQ CAEVQTDTRP WSGPYILRNQ DDREQWPRKF FNRTCKCTGN  
 101 FAGYNGGGCK FGWTGPDCNR KKPAIILRRNI HSLTAQEREQ FLGALDLAKK  
 151 SIHPDYVITT QHWLGLLGPN GTQPQIACNS YYDFFFVWLHY YSVRDTLLGP  
 201 GRPYKAIDFS HQGPAAFVTWH RYHLLWLERE LQRLLTGNESF ALPYWNFATG  
 251 KNECDVCTDD WLGAARQDDP TLISRNSRFS TWEIVCDSL DYNRRVTLCN  
 301 GTYEGLLRRN KVGRNNEKLP TLKNVQDCLS LQKFDSSPPFF QNSTFSFRNA  
 351 LEGFDKADGT LDSQVWMNLHN LAHSFLNGTN ALPHSAANDP VFVVLHSFTD  
 401 AIFDEWLKRN NPSTDAWPQE LAPIGHNRMY NMVPPFFPPVT NEELFLTAEQ  
 451 LGYNAYDLS EEEAPVWSTT LGVVIGILGA FVLLLGLLAF LQYRRLRKGY  
 501 APLMETGLSS KRYTEEA

FIG. 4

FIG. 4A

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## HUMAN TRP2

1 MSPLWWGFLL SCLGCKILPG AQGQFPRVCM TVDSL VNKEC CPRLGAE SAN  
51 VCGSQQGRGQ CTEVRADTRP WSGPYILRNQ DDRELWPRKF FHRTKCTGN  
101 FAGYNGCDCK FGWTGPNCER KKPPVIRQNI HSLSPQEREQ FLGALDLAKK  
151 RVHPDYVITT QHWLGLLGPN GTQPQFANC S VYDFFVWLHY YSVRDTLLGP  
201 GRPYRAIDFS HQGPAAFVTWH RYHLLCLERD LQRLIGNESF ALPYWNFATG  
251 RNECDVCTDQ LFGAARPDDP TLISRNSRFS SWETVCDSDL DYNHLYTLCN  
301 GTYEGLLRRN QMGRNSMCKLP TLKDIRDCLS LQKFDNPPFF QNSTFSFRNA  
351 LEGFDKADGT LDSQVMSLHN LVHSFLNGTN ALPHSAANDP IFVVLHSFTD  
401 AIFDEWMKRF NPPADAWPQE LAPIGHNRMY NMVPPFFPPVT NEELFLTSQD  
451 LGYSYAIIDL P VSVEETPGWP TTLLVVMGTL VALVGLFVLL AFLQYRRLRK  
501 GYTPLMETHL SSKRYTEEA

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Assay of CTLs Generated From  
Normal Donor PBL With Ad-GP100-  
Infected Dendritic Cells  
(Targets=T2 Cells Pulsed with GP100-  
F9 Peptide)

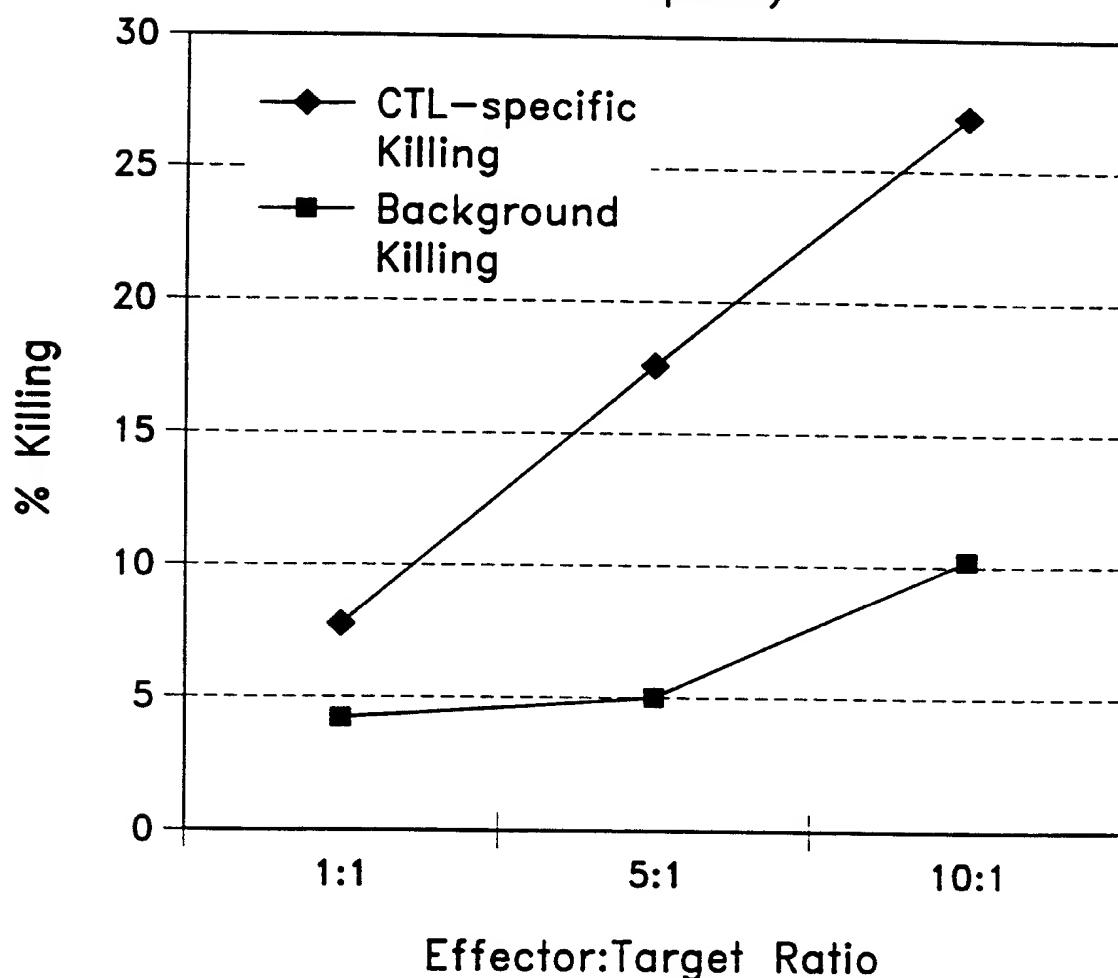


FIG. 5

## SEQUENCE LISTING

<110> Nicolette, Charles A.  
Genzyme Corporation

<120> COMPOSITIONS AND METHODS FOR ANTIGEN-SPECIFIC  
VACCINATION

<130> 159792001040

<140> Unassigned  
<141> 1999-03-19

<150> 60/078,890  
<151> 1998-03-20

<160> 17

<170> PatentIn Ver. 2.0

<210> 1  
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1 5 10 15

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20 25 30

Leu Gly Val Ser Arg Gln Leu Arg Thr Lys Ala Trp Asn Arg Gln Leu  
35 40 45

Tyr Pro Glu Trp Thr Glu Ala Gln Arg Leu Asp Cys Trp Arg Gly Gly  
50 55 60

Gln Val Ser Leu Lys Val Ser Asn Asp Gly Pro Thr Leu Ile Gly Ala  
65 70 75 80

Asn Ala Ser Phe Ser Ile Ala Leu Asn Phe Pro Gly Ser Gln Lys Val  
85 90 95

Leu Pro Asp Gly Gln Val Ile Trp Val Asn Asn Thr Ile Ile Asn Gly  
100 105 110

Ser Gln Val Trp Gly Gly Gln Pro Val Tyr Pro Gln Glu Thr Asp Asp  
115 120 125

Ala Cys Ile Phe Pro Asp Gly Gly Pro Cys Pro Ser Gly Ser Trp Ser  
130 135 140

Gln Lys Arg Ser Phe Val Tyr Val Trp Lys Thr Trp Gly Gln Tyr Trp  
145 150 155 160

Gln Val Leu Gly Gly Pro Val Ser Gly Leu Ser Ile Gly Thr Gly Arg  
165 170 175

Ala Met Leu Gly Thr His Thr Met Glu Val Thr Val Tyr His Arg Arg  
180 185 190

Gly Ser Arg Ser Tyr Val Pro Leu Ala His Ser Ser Ser Ala Phe Thr  
 195 200 205

Ile Thr Asp Gln Val Pro Phe Ser Val Ser Val Ser Gln Leu Arg Ala  
 210 215 220

Leu Asp Gly Gly Asn Lys His Phe Leu Arg Asn Gln Pro Leu Thr Phe  
 225 230 235 240

Ala Leu Gln Leu His Asp Pro Ser Gly Tyr Leu Ala Glu Ala Asp Leu  
 245 250 255

Ser Tyr Thr Trp Asp Phe Gly Asp Ser Ser Gly Thr Leu Ile Ser Arg  
 260 265 270

Ala Leu Val Val Thr His Thr Tyr Leu Glu Pro Gly Pro Val Thr Ala  
 275 280 285

Gln Val Val Leu Gln Ala Ala Ile Pro Leu Thr Ser Cys Gly Ser Ser  
 290 295 300

Pro Val Pro Gly Thr Thr Asp Gly His Arg Pro Thr Ala Glu Ala Pro  
 305 310 315 320

Asn Thr Thr Ala Gly Gln Val Pro Thr Thr Glu Val Val Gly Thr Thr  
 325 330 335

Pro Gly Gln Ala Pro Thr Ala Glu Pro Ser Gly Thr Thr Ser Val Gln  
 340 345 350

Val Pro Thr Thr Glu Val Ile Ser Thr Ala Pro Val Gln Met Pro Thr  
 355 360 365

Ala Glu Ser Thr Gly Met Thr Pro Glu Lys Val Pro Val Ser Glu Val  
 370 375 380

Met Gly Thr Thr Leu Ala Glu Met Ser Thr Pro Glu Ala Thr Gly Met  
 385 390 395 400

Thr Pro Ala Glu Val Ser Ile Val Val Leu Ser Gly Thr Thr Ala Ala  
 405 410 415

Gln Val Thr Thr Glu Trp Val Glu Thr Thr Ala Arg Glu Leu Pro  
 420 425 430

Ile Pro Glu Pro Glu Gly Pro Asp Ala Ser Ser Ile Met Ser Thr Glu  
 435 440 445

Ser Ile Thr Gly Ser Leu Gly Pro Leu Leu Asp Gly Thr Ala Thr Leu  
 450 455 460

Arg Leu Val Lys Arg Gln Val Pro Leu Asp Cys Val Leu Tyr Arg Tyr  
 465 470 475 480

Gly Ser Phe Ser Val Thr Leu Asp Ile Val Gln Gly Ile Glu Ser Ala  
 485 490 495

Glu Ile Leu Gln Ala Val Pro Ser Gly Glu Gly Asp Ala Phe Glu Leu  
 500 505 510

Thr Val Ser Cys Gln Gly Gly Leu Pro Lys Glu Ala Cys Met Glu Ile  
515 520 525

Ser Ser Pro Gly Cys Gln Pro Pro Ala Gln Arg Leu Cys Gln Pro Val  
530 535 540

Leu Pro Ser Pro Ala Cys Gln Leu Val Leu His Gln Ile Leu Lys Gly  
545 550 555 560

Gly Ser Gly Thr Tyr Cys Leu Asn Val Ser Leu Ala Asp Thr Asn Ser  
565 570 575

Leu Ala Val Val Ser Thr Gln Leu Ile Met Pro Gly Gln Glu Ala Gly  
580 585 590

Leu Gly Gln Val Pro Leu Ile Val Gly Ile Leu Leu Val Leu Met Ala  
595 600 605

Val Val Leu Ala Ser Leu Ile Tyr Arg Arg Arg Leu Met Lys Gln Asp  
610 615 620

Phe Ser Val Pro Gln Leu Pro His Ser Ser Ser His Trp Leu Arg Leu  
625 630 635 640

Pro Arg Ile Phe Cys Ser Cys Pro Ile Gly Glu Asn Ser Pro Leu Leu  
645 650 655

Ser Gly Gln Gln Val Xaa  
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Leu Glu Gly Ser  
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<400> 4  
Pro Leu Leu Pro  
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1 5

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23

<210> 7  
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cggcatcctg atcgtggtcc tggggattgc tctgcttatac ggctgctggc actgtagaag 120  
acgaagtgga tacagaacct tcatggacaa aaggcgtcat attggtattc aaaaaacctc 180  
aagggaaaga tgctcatgtg agagccctga tcaccaggac agccgactgt cttctcaaga 240  
gaaatccat cagccctgtgg ttcccaacgc tccgcctgcc tatgagaagc tctct 295

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24

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ctgctcatcg gctgttggtt ttgtagaaga cggaaatggat acagagcctt gatggataaa 180  
agtcttcatg ttggcactca atgtgcctta acaagaagat gcccacaaga agggtttgat 240  
catcgggaca gcaaagtgtc tcttcaagag aaaaactgtg aacctgtggc tcccaatgct 300

ccacctgctt atgagaaaact ctctgcagaa cagtcaccac caccttattc accttaa 357

<210> 10  
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1 5

<210> 11  
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1 5 10 15

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20 25 30

Ile Gly Cys Trp Tyr Cys Arg Arg Arg Ser Gly Tyr Arg Thr Leu Met  
35 40 45

Asp Lys Arg Arg His Ile Gly Ile Gln Lys Thr Ser Arg Glu Arg Cys  
50 55 60

Ser Cys Glu Ser Pro Asp His Gln Asp Ser Arg Leu Ser Ser Gln Glu  
65 70 75 80

Lys Ser His Gln Pro Val Val Pro Asn Ala Pro Pro Ala Tyr Glu Lys  
85 90 95

Leu Ser

<210> 12  
<211> 8  
<212> PRT  
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<400> 12  
Ser Pro Pro Pro Tyr Ser Pro Xaa  
1 5

<210> 13  
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<212> PRT  
<213> *Homo sapiens*

<400> 13  
Met Pro Arg Glu Asp Ala His Phe Ile Tyr Gly Tyr Pro Lys Lys Gly  
1 5 10 15

His Gly His Ser Tyr Thr Thr Ala Glu Ala Ala Gly Ile Gly Ile

|   |     |     |     |
|---|-----|-----|-----|
|   | 20  | 25  | 30  |
| Leu Thr Val Ile Leu Gly Val Leu Leu Leu Ile Gly Cys Trp Tyr Cys |     |     |     |
|   | 35  | 40  | 45  |
| Arg Arg Arg Asn Gly Tyr Arg Ala Leu Met Asp Lys Ser Leu His Val |     |     |     |
|   | 50  | 55  | 60  |
| Gly Thr Gln Cys Ala Leu Thr Arg Arg Cys Pro Gln Glu Gly Phe Asp |     |     |     |
|   | 65  | 70  | 75  |
| His Arg Asp Ser Lys Val Ser Leu Gln Glu Lys Asn Cys Glu Pro Val |     |     |     |
|   | 85  | 90  | 95  |
| Val Pro Asn Ala Pro Pro Ala Tyr Glu Lys Leu Ser Ala Glu Gln Ser |     |     |     |
|   | 100 | 105 | 110 |
| Pro Pro Pro Tyr Ser Pro Xaa                                     |     |     |     |
|   | 115 |     |     |

<210> 14  
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Met  
1

cca aga gaa gat gct cac ttc atc tat ggt tac ccc aag aag ggg cac 104  
Pro Arg Glu Asp Ala His Phe Ile Tyr Gly Tyr Pro Lys Lys Gly His  
5 10 15

```

ggc cac tct tac acc acg gct gaa gag gcc gct ggg atc ggc atc ctg 152
Gly His Ser Tyr Thr Thr Ala Glu Glu Ala Ala Gly Ile Gly Ile Leu
          20           25           30

```

```

aca gtg atc ctg gga gtc tta ctg ctc atc ggc tgt tgg tat tgt aga 200
Thr Val Ile Leu Gly Val Leu Leu Leu Ile Gly Cys Trp Tyr Cys Arg
35          40          45

```

```

aga cga aat gga tac aga gcc ttg atg gat aaa agt ctt cat gtt ggc 248
Arg Arg Asn Gly Tyr Arg Ala Leu Met Asp Lys Ser Leu His Val Gly
      50          55          60          65

```

```

act caa tgt gcc tta aca aga aga tgc cca caa gaa ggg ttt gat cat 296
Thr Gln Cys Ala Leu Thr Arg Arg Cys Pro Gln Glu Gly Phe Asp His
          70          75          80

```

```

cg  gac  agc  aaa  gtg  tct  ctt  caa  gag  aaa  aac  tgt  gaa  cct  gtg  gtt  344
Arg  Asp  Ser  Lys  Val  Ser  Leu  Gln  Glu  Lys  Asn  Cys  Glu  Pro  Val  Val
          85          90          95

```

ccc aat gct cca cct gct tat gag aaa ctc tct gca gaa cag tca cca 392  
Pro Asn Ala Pro Pro Ala Tyr Glu Lys Leu Ser Ala Glu Gln Ser Pro

|   |                     |      |
|---|---------------------|------|
| 100   | 105                 | 110  |
| cca cct tat tca cct taagagccag cgagacacac                           | gagacatgct gaaattat | ttt  |
| Pro Pro Tyr Ser Pro   |                     | 447  |
| 115   |                     |      |
| ctctcacact tttgcttgaa tttaatacag acatctaattt ttctcctttt gaaatgggtgt |                     |      |
| ggaaaaatgc aagccatctc taataataag tcagtgttaa aatttttagta ggtccgctag  |                     |      |
| cagtaataat catgtgagga aatgtgaga aatattaaat tggaaaaact ccatcaataa    |                     |      |
| atgttgcaat gcatgatact atctgtgcca gaggtatgt tagtaaatcc atgggtttat    |                     |      |
| tttctgagag acagaattca agtgggtatt ctggggccat ccaatttctc tttacttgaa   |                     |      |
| atttggctaa taacaaacta gtcaggtttt cgaaccttga ccgacatgaa ctgtacacag   |                     |      |
| aattgttcca gtactatgga gtgctcacaa aggatacttt tacaggttaa gacaaagggt   |                     |      |
| tgactggcct atttatctga tcaagaacat gtcagcaatg tctctttgtg ctctaaaatt   |                     |      |
| ctattatact acaataataat attgtaaaga tcctatactt cttttttttt gagatggagt  |                     |      |
| ttcgcttttgc ttgcccaggc tggagtgcaa tggcgcgatc ttggctcacc ataaccccg   |                     |      |
| cctcccaaggc tcaagcaatt ctccgcctt agcctcctga gtagctggta ttacaggcgt   |                     |      |
| gcgccactat gcctgactaa tttttagttt ttagtagaga cggggtttct ccatgttgg    |                     |      |
| caggctggc tcaaactcct gacctcaggt gatctcccg cctcagccctc ccaaagtgt     |                     |      |
| ggaattacag gcgtgagcca ccacgcctgg ctggatccta tatcttaggt aagacatata   |                     |      |
| acgcagtcta attacatttc acttcaaggc tcaatgctat tctaactaat gacaagtatt   |                     |      |
| ttctactaaa ccagaaattt gtagaaggat ttaaataagt aaaagctact atgtactgcc   |                     |      |
| tttagtgctga tgcctgtgtt ctgccttaaa tgtacctatg gcaatttagc tctcttgggt  |                     |      |
| tcccaaatcc ctctcacaag aatgtgcaga agaaatcata aaggatcaga gattctgaaa   |                     |      |
| aaaaaaaaaa aaaaaaaaaa aaaaaaaaaa aa                                 |                     |      |
|   |                     | 1559 |

<210> 15  
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 <213> Homo sapiens

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 1 5 10 15

His Gly His Ser Tyr Thr Ala Glu Glu Ala Ala Gly Ile Gly Ile  
 20 25 30

Leu Thr Val Ile Leu Gly Val Leu Leu Leu Ile Gly Cys Trp Tyr Cys  
 35 40 45

Arg Arg Arg Asn Gly Tyr Arg Ala Leu Met Asp Lys Ser Leu His Val

|    |    |    |
|----|----|----|
| 50 | 55 | 60 |
|----|----|----|

|     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| Gly | Thr | Gln | Cys | Ala | Leu | Thr | Arg | Arg | Cys | Pro | Gln | Glu | Gly | Phe | Asp |
| 65  |     |     |     |     |     |     |     |     |     |     |     |     |     |     | 80  |
|     |     |     |     | 70  |     |     |     |     | 75  |     |     |     |     |     |     |

|     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| His | Arg | Asp | Ser | Lys | Val | Ser | Leu | Gln | Glu | Lys | Asn | Cys | Glu | Pro | Val |
|     |     |     |     |     |     |     |     |     |     |     |     |     |     |     | 95  |
|     |     |     |     | 85  |     |     |     | 90  |     |     |     |     |     |     |     |

|     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| Val | Pro | Asn | Ala | Pro | Pro | Ala | Tyr | Glu | Lys | Leu | Ser | Ala | Glu | Gln | Ser |
|     |     |     |     |     |     |     |     |     |     |     |     |     |     |     | 110 |
|     |     |     |     | 100 |     |     |     | 105 |     |     |     |     |     |     |     |

|     |     |     |     |     |     |  |  |  |  |  |  |  |  |  |  |
|-----|-----|-----|-----|-----|-----|--|--|--|--|--|--|--|--|--|--|
| Pro | Pro | Pro | Tyr | Ser | Pro |  |  |  |  |  |  |  |  |  |  |
|     |     |     |     |     | 115 |  |  |  |  |  |  |  |  |  |  |

<210> 16

<211> 517

<212> PRT

<213> Mus Musculus

<400> 16

|     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |    |
|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|----|
| Met | Gly | Leu | Val | Gly | Trp | Gly | Leu | Leu | Gly | Cys | Leu | Gly | Cys | Gly |    |
| 1   |     |     |     |     |     |     |     |     |     |     |     |     |     |     | 15 |
|     |     |     |     | 5   |     |     |     | 10  |     |     |     |     |     |     |    |

|     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| Ile | Leu | Leu | Arg | Ala | Arg | Ala | Gln | Phe | Pro | Arg | Val | Cys | Met | Thr | Leu |
|     |     |     |     |     |     |     |     |     |     |     |     |     |     |     | 30  |
|     |     |     |     | 20  |     |     |     | 25  |     |     |     |     |     |     |     |

|     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| Asp | Gly | Val | Leu | Asn | Lys | Glu | Cys | Cys | Pro | Pro | Leu | Gly | Pro | Glu | Ala |
|     |     |     |     |     |     |     |     |     |     |     |     |     |     |     | 45  |
|     |     |     |     | 35  |     |     | 40  |     |     |     |     |     |     |     |     |

|     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| Thr | Asn | Ile | Cys | Gly | Phe | Leu | Glu | Gly | Arg | Gly | Gln | Cys | Ala | Glu | Val |
|     |     |     |     |     |     |     |     |     |     |     |     |     |     |     | 60  |
|     |     |     |     | 50  |     |     | 55  |     |     |     |     |     |     |     |     |

|     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| Gln | Thr | Asp | Thr | Arg | Pro | Trp | Ser | Gly | Pro | Tyr | Ile | Leu | Arg | Asn | Gln |
|     |     |     |     |     |     |     |     |     |     |     |     |     |     |     | 80  |
|     |     |     |     | 65  |     |     | 70  |     |     | 75  |     |     |     |     |     |

|     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| Asp | Asp | Arg | Glu | Gln | Trp | Pro | Arg | Lys | Phe | Phe | Asn | Arg | Thr | Cys | Lys |
|     |     |     |     |     |     |     |     |     |     |     |     |     |     |     | 95  |
|     |     |     |     | 85  |     |     | 90  |     |     |     |     |     |     |     |     |

|     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| Cys | Thr | Gly | Asn | Phe | Ala | Gly | Tyr | Asn | Cys | Gly | Gly | Cys | Lys | Phe | Gly |
|     |     |     |     |     |     |     |     |     |     |     |     |     |     |     | 110 |
|     |     |     |     | 100 |     |     | 105 |     |     |     |     |     |     |     |     |

|     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| Trp | Thr | Gly | Pro | Asp | Cys | Asn | Arg | Lys | Lys | Pro | Ala | Ile | Leu | Arg | Arg |
|     |     |     |     |     |     |     |     |     |     |     |     |     |     |     | 125 |
|     |     |     |     | 115 |     |     | 120 |     |     |     |     |     |     |     |     |

|     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| Asn | Ile | His | Ser | Leu | Thr | Ala | Gln | Glu | Arg | Glu | Gln | Phe | Leu | Gly | Ala |
|     |     |     |     |     |     |     |     |     |     |     |     |     |     |     | 140 |
|     |     |     |     | 130 |     |     | 135 |     |     |     |     |     |     |     |     |

|     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| Leu | Asp | Leu | Ala | Lys | Lys | Ser | Ile | His | Pro | Asp | Tyr | Val | Ile | Thr | Thr |
|     |     |     |     |     |     |     |     |     |     |     |     |     |     |     | 160 |
|     |     |     |     | 145 |     |     | 150 |     |     | 155 |     |     |     |     |     |

|     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| Gln | His | Trp | Leu | Gly | Leu | Leu | Gly | Pro | Asn | Gly | Thr | Gln | Pro | Gln | Ile |
|     |     |     |     |     |     |     |     |     |     |     |     |     |     |     | 175 |
|     |     |     |     | 165 |     |     | 170 |     |     |     |     |     |     |     |     |

|     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| Ala | Asn | Cys | Ser | Val | Tyr | Asp | Phe | Phe | Val | Trp | Leu | His | Tyr | Tyr | Ser |
|     |     |     |     |     |     |     |     |     |     |     |     |     |     |     | 190 |
|     |     |     |     | 180 |     |     | 185 |     |     |     |     |     |     |     |     |

|     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| Val | Arg | Asp | Thr | Leu | Leu | Gly | Pro | Gly | Arg | Pro | Tyr | Lys | Ala | Ile | Asp |
|     |     |     |     |     |     |     |     |     |     |     |     |     |     |     | 205 |
|     |     |     |     | 195 |     |     | 200 |     |     |     |     |     |     |     |     |

Phe Ser His Gln Gly Pro Ala Phe Val Thr Trp His Arg Tyr His Leu

|   |     |     |     |
|---|-----|-----|-----|
| 210   | 215 | 220 |     |
| Leu Trp Leu Glu Arg Glu Leu Gln Arg Leu Thr Gly Asn Glu Ser Phe |     |     |     |
| 225   | 230 | 235 | 240 |
| Ala Leu Pro Tyr Trp Asn Phe Ala Thr Gly Lys Asn Glu Cys Asp Val |     |     |     |
| 245   | 250 | 255 |     |
| Cys Thr Asp Asp Trp Leu Gly Ala Ala Arg Gln Asp Asp Pro Thr Leu |     |     |     |
| 260   | 265 | 270 |     |
| Ile Ser Arg Asn Ser Arg Phe Ser Thr Trp Glu Ile Val Cys Asp Ser |     |     |     |
| 275   | 280 | 285 |     |
| Leu Asp Asp Tyr Asn Arg Arg Val Thr Leu Cys Asn Gly Thr Tyr Glu |     |     |     |
| 290   | 295 | 300 |     |
| Gly Leu Leu Arg Arg Asn Lys Val Gly Arg Asn Asn Glu Lys Leu Pro |     |     |     |
| 305   | 310 | 315 | 320 |
| Thr Leu Lys Asn Val Gln Asp Cys Leu Ser Leu Gln Lys Phe Asp Ser |     |     |     |
| 325   | 330 | 335 |     |
| Pro Pro Phe Phe Gln Asn Ser Thr Phe Ser Phe Arg Asn Ala Leu Glu |     |     |     |
| 340   | 345 | 350 |     |
| Gly Phe Asp Lys Ala Asp Gly Thr Leu Asp Ser Gln Val Met Asn Leu |     |     |     |
| 355   | 360 | 365 |     |
| His Asn Leu Ala His Ser Phe Leu Asn Gly Thr Asn Ala Leu Pro His |     |     |     |
| 370   | 375 | 380 |     |
| Ser Ala Ala Asn Asp Pro Val Phe Val Val Leu His Ser Phe Thr Asp |     |     |     |
| 385   | 390 | 395 | 400 |
| Ala Ile Phe Asp Glu Trp Leu Lys Arg Asn Asn Pro Ser Thr Asp Ala |     |     |     |
| 405   | 410 | 415 |     |
| Trp Pro Gln Glu Leu Ala Pro Ile Gly His Asn Arg Met Tyr Asn Met |     |     |     |
| 420   | 425 | 430 |     |
| Val Pro Phe Phe Pro Pro Val Thr Asn Glu Glu Leu Phe Leu Thr Ala |     |     |     |
| 435   | 440 | 445 |     |
| Glu Gln Leu Gly Tyr Asn Tyr Ala Val Asp Leu Ser Glu Glu Ala     |     |     |     |
| 450   | 455 | 460 |     |
| Pro Val Trp Ser Thr Thr Leu Ser Val Val Ile Gly Ile Leu Gly Ala |     |     |     |
| 465   | 470 | 475 | 480 |
| Phe Val Leu Leu Leu Gly Leu Leu Ala Phe Leu Gln Tyr Arg Arg Leu |     |     |     |
| 485   | 490 | 495 |     |
| Arg Lys Gly Tyr Ala Pro Leu Met Glu Thr Gly Leu Ser Ser Lys Arg |     |     |     |
| 500   | 505 | 510 |     |
| Tyr Thr Glu Glu Ala   |     |     |     |
| 515   |     |     |     |

<211> 519  
 <212> PRT  
 <213> Homo sapiens

<400> 17

|     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| Met | Ser | Pro | Leu | Trp | Trp | Gly | Phe | Leu | Leu | Ser | Cys | Leu | Gly | Cys | Lys |
| 1   |     |     |     | 5   |     |     |     | 10  |     |     |     |     |     | 15  |     |

Ile Leu Pro Gly Ala Gln Gly Gln Phe Pro Arg Val Cys Met Thr Val

|  |    |  |  |  |    |  |  |  |  |    |  |  |  |  |  |
|--|----|--|--|--|----|--|--|--|--|----|--|--|--|--|--|
|  | 20 |  |  |  | 25 |  |  |  |  | 30 |  |  |  |  |  |
|--|----|--|--|--|----|--|--|--|--|----|--|--|--|--|--|

Asp Ser Leu Val Asn Lys Glu Cys Cys Pro Arg Leu Gly Ala Glu Ser

|  |    |  |  |    |  |  |  |    |  |  |  |  |  |  |  |
|--|----|--|--|----|--|--|--|----|--|--|--|--|--|--|--|
|  | 35 |  |  | 40 |  |  |  | 45 |  |  |  |  |  |  |  |
|--|----|--|--|----|--|--|--|----|--|--|--|--|--|--|--|

Ala Asn Val Cys Gly Ser Gln Gln Gly Arg Gly Gln Cys Thr Glu Val

|  |    |  |    |  |  |    |  |  |  |  |  |  |  |  |  |
|--|----|--|----|--|--|----|--|--|--|--|--|--|--|--|--|
|  | 50 |  | 55 |  |  | 60 |  |  |  |  |  |  |  |  |  |
|--|----|--|----|--|--|----|--|--|--|--|--|--|--|--|--|

Arg Ala Asp Thr Arg Pro Trp Ser Gly Pro Tyr Ile Leu Arg Asn Gln

|  |    |  |    |  |    |  |  |    |  |  |  |  |  |  |  |
|--|----|--|----|--|----|--|--|----|--|--|--|--|--|--|--|
|  | 65 |  | 70 |  | 75 |  |  | 80 |  |  |  |  |  |  |  |
|--|----|--|----|--|----|--|--|----|--|--|--|--|--|--|--|

Asp Asp Arg Glu Leu Trp Pro Arg Lys Phe Phe His Arg Thr Cys Lys

|  |    |  |  |    |  |  |    |  |  |  |  |  |  |  |  |
|--|----|--|--|----|--|--|----|--|--|--|--|--|--|--|--|
|  | 85 |  |  | 90 |  |  | 95 |  |  |  |  |  |  |  |  |
|--|----|--|--|----|--|--|----|--|--|--|--|--|--|--|--|

Cys Thr Gly Asn Phe Ala Gly Tyr Asn Cys Gly Asp Cys Lys Phe Gly

|  |     |  |     |  |  |     |  |  |  |  |  |  |  |  |  |
|--|-----|--|-----|--|--|-----|--|--|--|--|--|--|--|--|--|
|  | 100 |  | 105 |  |  | 110 |  |  |  |  |  |  |  |  |  |
|--|-----|--|-----|--|--|-----|--|--|--|--|--|--|--|--|--|

Trp Thr Gly Pro Asn Cys Glu Arg Lys Lys Pro Pro Val Ile Arg Gln

|  |     |  |     |  |     |  |  |  |  |  |  |  |  |  |  |
|--|-----|--|-----|--|-----|--|--|--|--|--|--|--|--|--|--|
|  | 115 |  | 120 |  | 125 |  |  |  |  |  |  |  |  |  |  |
|--|-----|--|-----|--|-----|--|--|--|--|--|--|--|--|--|--|

Asn Ile His Ser Leu Ser Pro Gln Glu Arg Glu Gln Phe Leu Gly Ala

|  |     |  |     |  |     |  |  |  |  |  |  |  |  |  |  |
|--|-----|--|-----|--|-----|--|--|--|--|--|--|--|--|--|--|
|  | 130 |  | 135 |  | 140 |  |  |  |  |  |  |  |  |  |  |
|--|-----|--|-----|--|-----|--|--|--|--|--|--|--|--|--|--|

Leu Asp Leu Ala Lys Lys Arg Val His Pro Asp Tyr Val Ile Thr Thr

|  |     |  |     |  |     |  |  |     |  |  |  |  |  |  |  |
|--|-----|--|-----|--|-----|--|--|-----|--|--|--|--|--|--|--|
|  | 145 |  | 150 |  | 155 |  |  | 160 |  |  |  |  |  |  |  |
|--|-----|--|-----|--|-----|--|--|-----|--|--|--|--|--|--|--|

Gln His Trp Leu Gly Leu Leu Gly Pro Asn Gly Thr Gln Pro Gln Phe

|  |     |  |     |  |     |  |  |  |  |  |  |  |  |  |  |
|--|-----|--|-----|--|-----|--|--|--|--|--|--|--|--|--|--|
|  | 165 |  | 170 |  | 175 |  |  |  |  |  |  |  |  |  |  |
|--|-----|--|-----|--|-----|--|--|--|--|--|--|--|--|--|--|

Ala Asn Cys Ser Val Tyr Asp Phe Phe Val Trp Leu His Tyr Tyr Ser

|  |     |  |     |  |     |  |  |  |  |  |  |  |  |  |  |
|--|-----|--|-----|--|-----|--|--|--|--|--|--|--|--|--|--|
|  | 180 |  | 185 |  | 190 |  |  |  |  |  |  |  |  |  |  |
|--|-----|--|-----|--|-----|--|--|--|--|--|--|--|--|--|--|

Val Arg Asp Thr Leu Leu Gly Pro Gly Arg Pro Tyr Arg Ala Ile Asp

|  |     |  |     |  |     |  |  |  |  |  |  |  |  |  |  |
|--|-----|--|-----|--|-----|--|--|--|--|--|--|--|--|--|--|
|  | 195 |  | 200 |  | 205 |  |  |  |  |  |  |  |  |  |  |
|--|-----|--|-----|--|-----|--|--|--|--|--|--|--|--|--|--|

Phe Ser His Gln Gly Pro Ala Phe Val Thr Trp His Arg Tyr His Leu

|  |     |  |     |  |     |  |  |  |  |  |  |  |  |  |  |
|--|-----|--|-----|--|-----|--|--|--|--|--|--|--|--|--|--|
|  | 210 |  | 215 |  | 220 |  |  |  |  |  |  |  |  |  |  |
|--|-----|--|-----|--|-----|--|--|--|--|--|--|--|--|--|--|

Leu Cys Leu Glu Arg Asp Leu Gln Arg Leu Ile Gly Asn Glu Ser Phe

|  |     |  |     |  |     |  |     |  |  |  |  |  |  |  |  |
|--|-----|--|-----|--|-----|--|-----|--|--|--|--|--|--|--|--|
|  | 225 |  | 230 |  | 235 |  | 240 |  |  |  |  |  |  |  |  |
|--|-----|--|-----|--|-----|--|-----|--|--|--|--|--|--|--|--|

Ala Leu Pro Tyr Trp Asn Phe Ala Thr Gly Arg Asn Glu Cys Asp Val

|  |     |  |     |  |     |  |  |  |  |  |  |  |  |  |  |
|--|-----|--|-----|--|-----|--|--|--|--|--|--|--|--|--|--|
|  | 245 |  | 250 |  | 255 |  |  |  |  |  |  |  |  |  |  |
|--|-----|--|-----|--|-----|--|--|--|--|--|--|--|--|--|--|

Cys Thr Asp Gln Leu Phe Gly Ala Ala Arg Pro Asp Asp Pro Thr Leu

|  |     |  |     |  |     |  |  |  |  |  |  |  |  |  |  |
|--|-----|--|-----|--|-----|--|--|--|--|--|--|--|--|--|--|
|  | 260 |  | 265 |  | 270 |  |  |  |  |  |  |  |  |  |  |
|--|-----|--|-----|--|-----|--|--|--|--|--|--|--|--|--|--|

Ile Ser Arg Asn Ser Arg Phe Ser Ser Trp Glu Thr Val Cys Asp Ser

|  |     |  |     |  |     |  |  |  |  |  |  |  |  |  |  |
|--|-----|--|-----|--|-----|--|--|--|--|--|--|--|--|--|--|
|  | 275 |  | 280 |  | 285 |  |  |  |  |  |  |  |  |  |  |
|--|-----|--|-----|--|-----|--|--|--|--|--|--|--|--|--|--|

Leu Asp Asp Tyr Asn His Leu Val Thr Leu Cys Asn Gly Thr Tyr Glu

|  |     |  |     |  |     |  |  |  |  |  |  |  |  |  |  |
|--|-----|--|-----|--|-----|--|--|--|--|--|--|--|--|--|--|
|  | 290 |  | 295 |  | 300 |  |  |  |  |  |  |  |  |  |  |
|--|-----|--|-----|--|-----|--|--|--|--|--|--|--|--|--|--|

Gly Leu Leu Arg Arg Asn Gln Met Gly Arg Asn Ser Met Lys Leu Pro  
305 310 315 320

Thr Leu Lys Asp Ile Arg Asp Cys Leu Ser Leu Gln Lys Phe Asp Asn  
325 330 335

Pro Pro Phe Phe Gln Asn Ser Thr Phe Ser Phe Arg Asn Ala Leu Glu  
340 345 350

Gly Phe Asp Lys Ala Asp Gly Thr Leu Asp Ser Gln Val Met Ser Leu  
355 360 365

His Asn Leu Val His Ser Phe Leu Asn Gly Thr Asn Ala Leu Pro His  
370 375 380

Ser Ala Ala Asn Asp Pro Ile Phe Val Val Leu His Ser Phe Thr Asp  
385 390 395 400

Ala Ile Phe Asp Glu Trp Met Lys Arg Phe Asn Pro Pro Ala Asp Ala  
405 410 415

Trp Pro Gln Glu Leu Ala Pro Ile Gly His Asn Arg Met Tyr Asn Met  
420 425 430

Val Pro Phe Phe Pro Pro Val Thr Asn Glu Glu Leu Phe Leu Thr Ser  
435 440 445

Asp Gln Leu Gly Tyr Ser Tyr Ala Ile Asp Leu Pro Val Ser Val Glu  
450 455 460

Glu Thr Pro Gly Trp Pro Thr Thr Leu Leu Val Val Met Gly Thr Leu  
465 470 475 480

Val Ala Leu Val Gly Leu Phe Val Leu Leu Ala Phe Leu Gln Tyr Arg  
485 490 495

Arg Leu Arg Lys Gly Tyr Thr Pro Leu Met Glu Thr His Leu Ser Ser  
500 505 510

Lys Arg Tyr Thr Glu Glu Ala  
515

## INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US99/06034

## A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :Please See Extra Sheet.

US CL :Please See Extra Sheet.

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 424/184.1, 277.1, 287.1, 93.2, 93.71; 435/372, 373; 514/2, 44; 530/350, 806, 827, 828; 536/23.1, 23.5

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

DIALOG medicine index, APS, WEST

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

| Category* | Citation of document, with indication, where appropriate, of the relevant passages   | Relevant to claim No. |
|-----------|--|-----------------------|
| A, P      | LIU, M. Transfected human dendritic cells as cancer vaccines. Nat. Biotech. April 1988, Vol. 16, pages 335-336, see entire document. | 1-23                  |
| A         | DONNELLY, J.J. et al. DNA vaccines. Ann. Rev. Immunol. 1997, Vol. 15, pages 617-648, see entire document.                            | 1-23                  |
| A, P      | PARDOLL, D.M. Cancer vaccines. Nature Medicine. May 1998, Vol. 4, No.5(suppl.), pages 525-531, see entire document.                  | 1-23                  |

 Further documents are listed in the continuation of Box C.  See patent family annex.

|   |     |  |
|---|-----|--|
| * Special categories of cited documents:  | *T* | later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention  |
| *A* document defining the general state of the art which is not considered to be of particular relevance  | *X* | document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone   |
| *E* earlier document published on or after the international filing date  | *Y* | document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art |
| *L* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) | *&* | document member of the same patent family  |
| *O* document referring to an oral disclosure, use, exhibition or other means  |     |  |
| *P* document published prior to the international filing date but later than the priority date claimed  |     |  |

Date of the actual completion of the international search

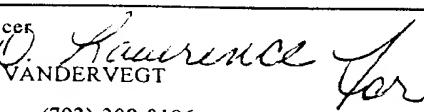
29 JUNE 1999

Date of mailing of the international search report

03 AUG 1999

Name and mailing address of the ISA/US  
Commissioner of Patents and Trademarks  
Box PCT  
Washington, D.C. 20231

Facsimile No. (703) 305-3230

Authorized officer  
F PIERRE VANDERVEGT  
Telephone No. (703) 308-0196  


## INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US99/06034

## C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

| Category*    | Citation of document, with indication, where appropriate, of the relevant passages  | Relevant to claim No.    |
|--------------|---|--------------------------|
| X            | BOCZKOWSKI, D. et al. Dendritic cells pulsed with RNA are potent antigen-presenting cells in vitro and in vivo. J. Exp. Med. 01 August 1996, Vol. 184, No. 2, pages 465-472, see entire document, abstract in particular. | 1-3, 7-10, 14-17, 20, 22 |
| X            | US 5,679,647 A (CARSON et al) 21 October 1997, see entire document.   | 1-3, 13, 20-23           |
| X, P<br>---- | US 5,844,075 A (KAWAKAMI et al) 01 December 1998, see entire document.  | 1, 13, 20, 22-23<br>---- |
| Y, P         |   | 2-12, 14-19, 21          |

**INTERNATIONAL SEARCH REPORT**

International application No.  
PCT/US99/06034

**A. CLASSIFICATION OF SUBJECT MATTER:**  
IPC (6):

A01N 37/18, 43/04, 63/00; A61K 31/70, 35/12, 35/36, 39/00; C12N 5/02, 5/06; C07K 1/00; C07H 21/02, 21/04

**A. CLASSIFICATION OF SUBJECT MATTER:**  
US CL :

424/184.1, 277.1, 287.1, 93.2, 93.71; 435/372, 373; 514/2, 44; 530/350, 806, 827, 828; 536/23.1, 23.5